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CARCINOMATOUS TRANSFORMATION IN A TRANSPLANTABLE RAT FIBROADENOMA.

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Received for publication February 13, 1942.

IN a previous communication (Selbie, 1941) a description was given of the experimental and histological findings in a transplantable rat fibroadenoma which was obtained by the author from a spontaneous tumour in 1934. Both the epithelial and fibrous components of this tumour frequently showed structural alterations which could be largely ascribed to hormonal influences. In the fibrous component the most important change was the appearance of new sarcomatous tumours but, although the epithelium showed great variations in structure and arrangement, no changes suggestive of carcinoma had been observed up to the date of publication. Carcinoma is now appearing in one transplantation line of this tumour and these new changes will be described here.

EXPERIMENTAL.

The substrain in which carcinomatous changes are appearing was derived from the tumour in the left flank of Rat 5, Passage 5A (Selbie, 1941, Fig. 1). This female rat was killed 111 weeks after inoculation, and examination of the tumour showed that it was mostly fibroadenomatous, but that a new connective-tissue tumour was present in one part. Fibroadenomatous grafts were inoculated into the left flanks and grafts of the fibromatous tissue were inoculated into the right flanks of the rats in Passage 6F (Fig. 1). Tumours developed only in one male rat, and at 17 weeks grafts from each tumour were inoculated into the corresponding flanks of the rats in Passage 7E. In later passages the rats were inoculated in both flanks with material derived from the fibroadenomatous tumour in Passage 6F. The number and sex of the inoculated rats, the yield of tumours and the incidence of carcinomatous changes in this substrain are shown in Fig. 1. The growth and history of the tumours in the passages where carcinomatous tumours appeared are shown graphically

in Fig. 2. The fibromatous tumours in Passages 6F and 7E are omitted from both figures.

It can be seen in Fig. 2 that the growth characters of this substrain of the rat fibroadenoma are very similar to those of the earlier passages (Selbie, 1941, Fig. 2), in that there is usually a latent period of many weeks followed by growth that is usually very slow, but may vary greatly even in pairs of tumours in one rat. There is, however, a shortening of the average period between inoculation and the formation of a palpable tumour from 20 weeks in the earlier passages to 11 weeks, and there is also a greater yield of tumours for, in spite

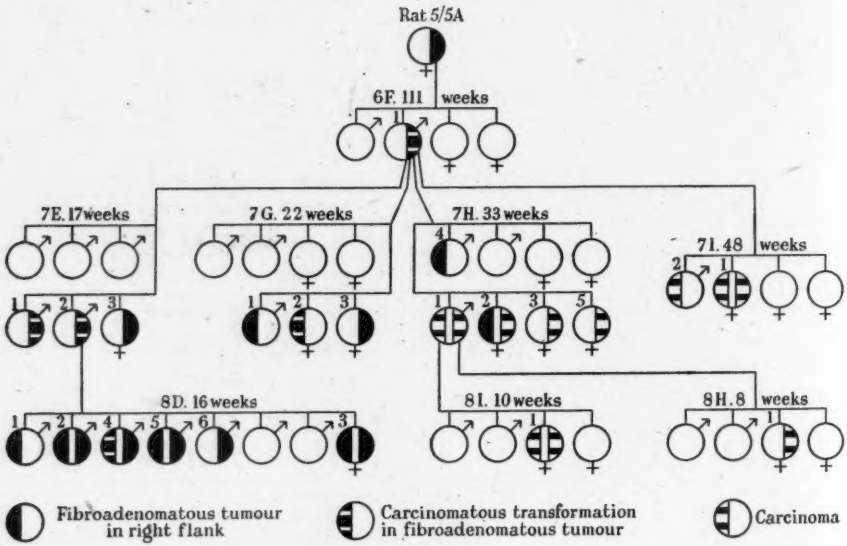


FIG. 1.—Results of transplantation of the carcinomatous rat fibroadenoma substrain.

of the higher proportion of males, the percentage of successfully inoculated rats is 50, whereas it was formerly 39 in all rats.

It will also be seen in Fig. 2 that the tumours in Rat 1, Passage 6F, in Rats 1 and 2, Passage 7E, in Rat 2, Passage 7G, and in the right flank of Rat 4, Passage 8D, show expansions in growth rate at intervals after inoculation of 27, 46, 30, 33 and 14 weeks respectively. It was noted in the earlier communication on this fibroadenoma that similar increases in growth, which occurred at a much later stage after inoculation, were associated with sarcomatous changes. The changes described here are also in the direction of malignancy, but, as is indicated in Fig. 1, they concern the glandular component of the tumour.

The final effects of the tumour in Rat 1, Passage 6F, and the results of transplantation confirm the nature of the change in this tumour. At 54 weeks a glandular metastasis was palpated in the left axilla and the rat died at

56 weeks with metastases in the lungs, in the lymphatic glands of the left axilla and groin, in mediastinal and retroperitoneal glands and in the pericardium and parietal pleura (Fig. 7). When Passage 7H was made at 33 weeks, that is six weeks after an acceleration in growth was detected, it was noted that part of the biopsy specimen was fibroadenomatous with large cysts and

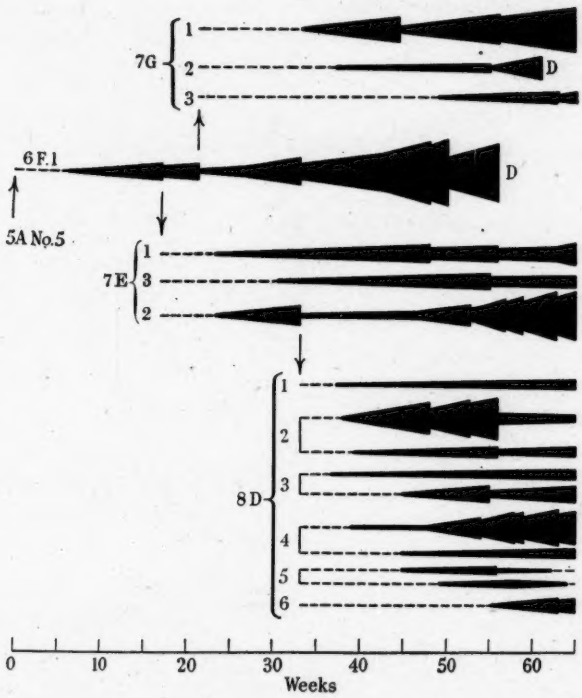


FIG. 2.—Growth of the tumours in the carcinomatous rat fibroadenoma substrain. Where two tumours in one rat are shown, the upper and lower expanding triangles represent the tumours in the right and left flanks respectively. The apex of each triangle represents the time at which the tumour became palpable and the subsequent width of the triangle at any point is 1/4 of the long axis of the tumour at the number of weeks indicated. An interruption in a triangle represents partial removal of the tumour by operation. The death of a rat is represented by the letter "D." Note the accelerations in growth in the tumours in Rat 1, Passage 6F, at 27 weeks, in Rats 1 and 2, Passage 7E, at 46 and 30 weeks, in Rat 2, Passage 7G, at 33 weeks, and in Rat 4, Passage 8D, at 14 weeks after inoculation.

that the remainder was firm and solid in structure (Fig. 5). In transplanting this tumour an attempt was made to separate the two parts with the result shown in Passage 7H (Fig. 1). In Rat 1 carcinomata grew rapidly in both flanks and killed the rat in 20 weeks with metastases in the lungs, and in the inguinal, axillary and retroperitoneal glands. In the other rats, which were

apparently successfully inoculated in opposite flanks with the fibroadenomatous and carcinomatous material, there were one slow-growing carcinoma in Rat 2, two temporary tumours, probably carcinomata, in Rats 3 and 5, and two fibroadenomatous tumours in Rats 2 and 4. The later passages of carcinomatous tumours shown in Fig. 1 yielded progressive tumours only in Rat 1, Passage 8I, which died at 18 weeks with generalized metastases.

None of the other four tumours that showed a sudden increase in growth rate has produced palpable metastases. One rat, 7G, No. 2, died without metastases six weeks after the increased growth rate was detected, and the other three are still within the period of observation during which metastases may be expected to appear as judged by the history of Rat 1, Passage 6F.

HISTOLOGICAL.

The part of the tumour from Rat 5, Passage 5A, that provided the material for Passage 6F shows a variety of epithelial changes, including the formation of cysts, some lined by vacuolated secretory cells, some by flattened epithelium, and others by epithelium several layers deep (Fig. 3). There are also areas

FIG. 3.—Parent tumour of the carcinomatous substrain from the left flank of Rat 5, Passage 5A, after 111 weeks' growth in a female rat. Irregular acinar proliferation can be seen particularly in the lower part of the figure. A variety of cysts are present, most of which are lined by several layers of cells. A higher magnification of another field of this tumour is shown in Fig. 6 of the previous communication (Selbie, 1941). × 33.

FIG. 4.—Tumour from Rat 1, Passage 6F, at 17 weeks. The proliferative character of the epithelium in the parent tumour (Fig. 3) is maintained. The connective tissue is abundant, well differentiated, and arranged concentrically round the glandular tissue. × 75.

FIG. 5.—The same tumour at 33 weeks. The tumour now shows large cysts and is being invaded and replaced by carcinoma. × 16.

FIG. 6.—A higher magnification of part of Fig. 5. A tubule is surrounded by the advancing edge of the carcinoma which is invading the fibrous tissue of the fibroadenoma. × 230.

FIG. 7.—Rat 1, Passage 6F, 56 weeks after inoculation. There are metastases in the left axilla, mediastinum, pericardium and lungs.

FIG. 8.—Metastasis in a retroperitoneal lymph gland from Rat 1, Passage 6F, at 56 weeks. Acinar differentiation is shown in the central part of the metastasis. The periphery shows a vigorous fibroblastic reaction. × 60.

FIG. 9.—Tumour from Rat 1, Passage 7E, at 31 weeks. There is irregular acinar proliferation with the formation of structures not unlike intraductal papillomata. The fibrous stroma is greatly reduced and there is considerable infiltration by inflammatory cells. × 65.

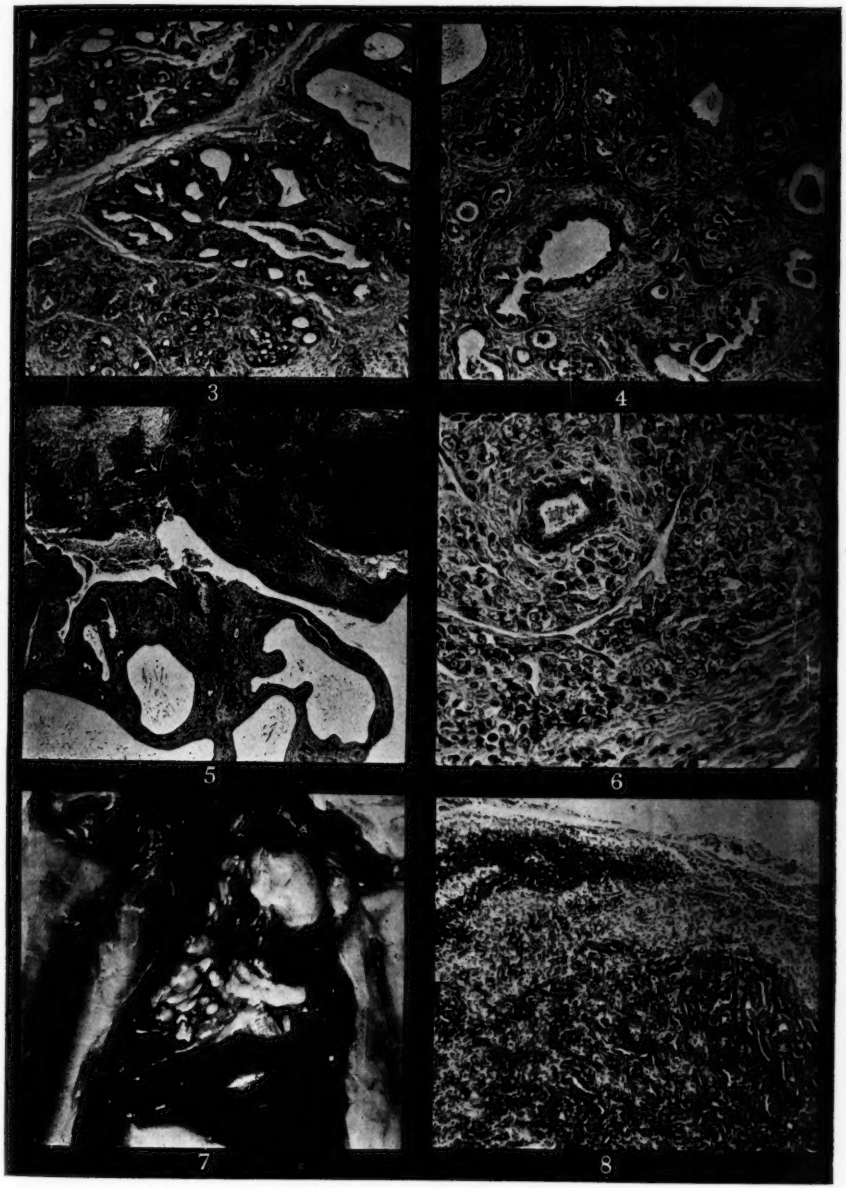
FIG. 10.—The same tumour at 48 weeks. Undifferentiated carcinoma has now appeared. There is a heavy infiltration by inflammatory cells confined to the periphery of the carcinomatous nodules. × 75.

FIG. 11.—Tumour from Rat 2, Passage 7E, at 16 weeks. There is irregular epithelial proliferation with the formation of thick cords and occasional cystic acini. The fibrous tissue is well developed and maintains its concentric arrangement. × 95.

FIG. 12.—The same tumour at 40 weeks. Epithelial proliferation has continued with the formation of smaller glandular elements and the fibrous tissue is greatly reduced and infiltrated by inflammatory cells. In the central and upper parts of the figure the epithelium presents the appearance of undifferentiated carcinoma with very little stroma. × 95.

FIG. 13.—Tumour from Rat 2, Passage 7G, at 39 weeks. This part of the tumour consists of irregular papilliform epithelial growth with a scanty and very vascular stroma. Part of the fibroadenomatous periphery of the tumour is shown. × 95.

FIG. 14.—Tumour from Rat 4, Passage 8D, at 25 weeks. The tumour consists of solid masses of epithelial cells demarcated by a fine vascular stroma. Some attempt at acinar differentiation can be seen. × 85.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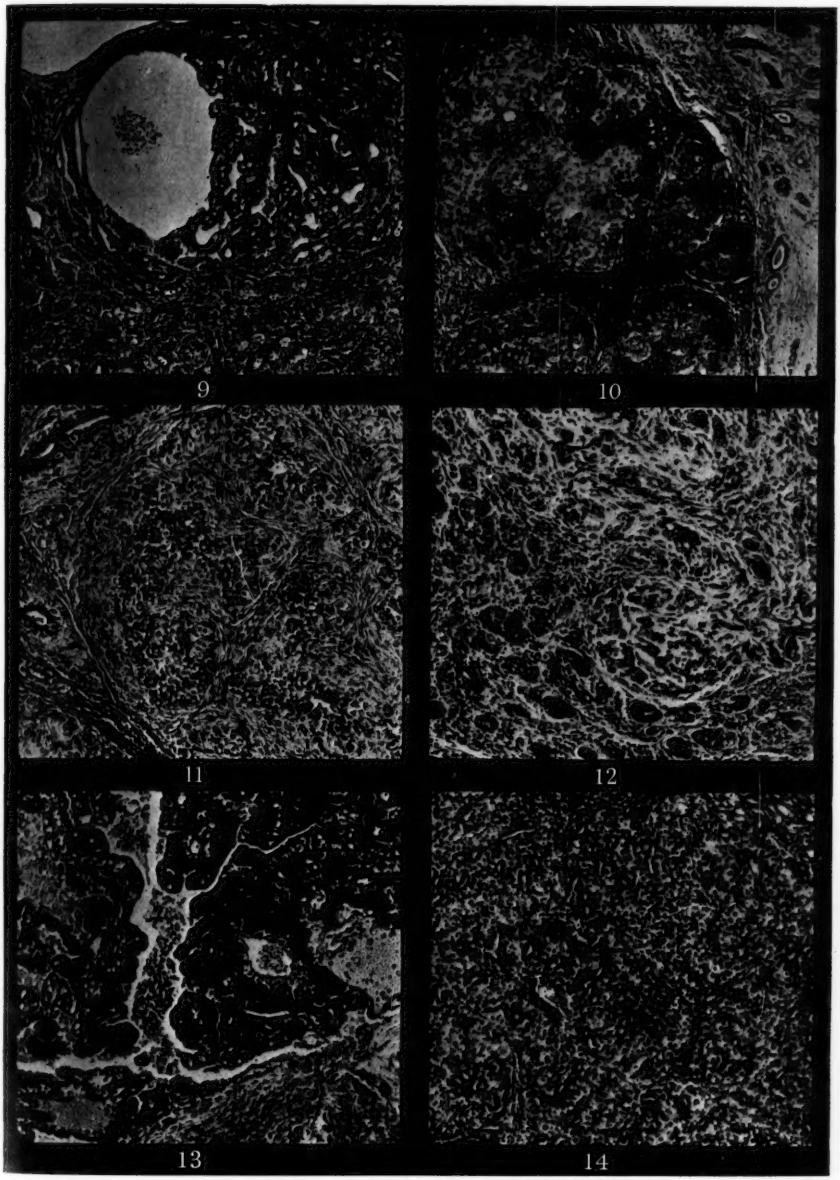
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of irregular acinar proliferation, but no evidence of carcinomatous changes can be found. These proliferative changes in the epithelium, which can be attributed to the effects of hormonal stimulation during the tumour's 111 weeks' growth in a female rat (Selbie, 1941), are continued in the passage tumours, although most of them are growing in male rats.

In the tumour in Rat 1, Passage 6F, at 17 weeks after inoculation, most of the acini and cysts are lined by several layers of cells, and under a higher magnification a considerable number of mitoses can be seen (Fig. 4). The connective tissue, however, is more abundant than in the previous tumour, and is highly differentiated and arranged concentrically round the glandular tissue as in earlier passages. In most of the remaining tumours of this substrain the glandular tissue consists mainly of solid cords of epithelial cells many layers thick. Acinar differentiation of the type shown in Fig. 4 is present in most tumours and is the predominating feature in some. Epithelial mitoses are numerous and some irregularity in arrangement is frequent. Cystic acini are often present, and the formation of large cysts is a prominent feature in the tumours in Rat 1, Passage 6F, at 33 weeks (Fig. 5), and in Rat 1, Passage 7G. Formations similar in structure to intraductal papillomata have also been observed in the tumours in Rats 1 and 3 of Passage 7E (Fig. 9), and are of interest since they appear to be precursors of the epithelial tumours to be described in Rat 2, Passage 7G, and in Rat 4, Passage 8D. Several tumours show small areas suggestive of carcinoma but, up to the present, they have the general appearance of fibroadenomata with an abundant and well differentiated fibrous component. On the other hand, in the five tumours that have developed an increased growth rate the predominant histological feature is epithelial proliferation outstripping the fibrous tissue.

Carcinomatous Transformation.

Rat 1, *Passage* 6F.—The appearance of the tumour in this rat at 17 weeks has already been described (Fig. 4). A section of a biopsy specimen at 22 weeks shows no essential difference, but at 33 weeks, that is, 6 weeks after an accerelation in growth was noted, the original tumour, now showing large cysts, is being invaded and replaced by carcinoma (Fig. 5). The junction of fibroadenoma and carcinoma has been largely lost in the preparation, but at one point a few tubules of the fibroadenoma can be seen embedded in the carcinoma. One of these tubules surrounded by the advancing edge of the carcinoma is shown in Fig. 6. A later section of this tumour at 48 weeks shows that the fibroadenoma has been completely replaced by carcinoma. The development of metastases in this rat (Fig. 7) confirms the malignant nature of this tumour, and its epithelial origin is shown by the acinar differentiation that can be seen in older parts of the primary growth, in metastases (Fig. 8) and in transplanted tumours.

Rat 1, *Passage* 7E.—A section from the tumour in this rat at 31 weeks shows two distinct types of growth. One part shows a structure similar to that of the tumour in Rat 1, Passage 6F, at 17 weeks (Fig. 4), with numerous cystic acini and an abundant fibrous stroma. In the other part the glandular tissue predominates, forming an adenomatous tumour with areas of a type of intracystic epithelial proliferation that has already been mentioned

(Fig. 9). The fibrous tissue is much reduced and heavily infiltrated by inflammatory cells. These features are again seen in a biopsy specimen taken at 39 weeks after a further 8 weeks of slow growth. The third biopsy was made 2 weeks after more rapid growth was detected, and, as in the tumour in Rat 1, Passage 6F, a section shows that part of the tumour is now formed of solid nodules of undifferentiated carcinoma (Fig. 10). A heavy infiltration by inflammatory cells is also present, and it can be seen in Fig. 10 that this is confined to the periphery of the carcinomatous nodules.

Rat 2, *Passage* 7E.—In the tumour from this rat at 16 weeks after inoculation the glandular tissue consists mostly of cords of cells many layers thick with occasional cystic acini (Fig. 11). The connective tissue is well developed and arranged concentrically round the epithelial tissue. At 36 weeks, that is, 6 weeks after the change in growth rate was observed, a section shows in one part of the tumour two small areas of irregular epithelial growth presenting the appearance of carcinoma, but with very few mitoses. A later section at 40 weeks shows that part of the tumour consists almost entirely of irregular proliferating cords of epithelial cells in a cellular and much reduced stroma. In some areas the stroma is very scanty, with the result that the tumour assumes the appearance of an undifferentiated carcinoma (Fig. 12). A notable feature of this tumour is the high degree of infiltration by inflammatory cells. Material from a more extensive biopsy made 2 weeks later shows that part of the tumour retains its early adenomatous appearance, and is provided with a cellular stroma with large numbers of infiltrating inflammatory cells.

Rat 2, *Passage* 7G.—A section taken from the tumour in this rat at 33 weeks, and immediately before an acceleration in growth was noted, shows an adenomatous tumour with numerous acini and a stroma that is more cellular and of more open texture than usual. The stroma is also infiltrated by numerous inflammatory cells. Six weeks later it was noted that the tumour consisted of a shell of firm tissue and a very vascular friable core. A section shows that the shell consists of fibroadenomatous tissue, and that the core is an actively growing papilliform tumour showing numerous mitoses and with a scanty but very vascular stroma (Fig. 13).

Rat 4, *Passage* 8D.—The first biopsy specimen of the tumour in the right flank of this rat was taken 21 weeks after inoculation and 7 weeks after an increase in growth rate was detected. In general structure it is similar to the tumour in Rat 2, Passage 7G, having a firm shell and a soft vascular centre. The fibroadenomatous shell, like the tumour from which Passage 8D was made, consists of thick adenomatous cords with a well-developed stroma (*cf.* Fig. 11). The central epithelial tumour is a papillary type of growth consisting of irregular acini with more solid areas of undifferentiated cells adjoining a fine vascular stroma. Another section at 25 weeks shows a part of the tumour which has retained this general structure, but has a solid and undifferentiated appearance characteristic of carcinoma (Fig. 14).

DISCUSSION.

In contrast to sarcomatous transformation, which has been frequently observed in transplantable rat fibroadenomata, carcinomatous changes in

these tumours seem to be rare. Emge (1938), in a description of one of his transplantable rat fibroadenomata, has noted carcinomatous changes characterized by hyperplasia of glandular components with invasion of surrounding stroma, but on transplantation he obtained differentiated fibroadenoma. The changes described here, however, appear to be irreversible, and in one tumour have proceeded to the formation of metastases in the original rat and in transplanted rats. It may therefore be presumed that some exceptional factor has produced this cancerous strain in a fibroadenoma which in previous passages and in other transplantation lines has shown no tendency towards manifestly malignant growth.

It has been shown that an acceleration in growth in tumours of this substrain is associated with the appearance of independent epithelial neoplasia which has the histological characteristics of carcinoma. In other tumours which have the general appearance and growth characters of fibroadenomata there are areas which are histologically malignant. It can also be seen in Figs. 9 and 11 that malignant characters are present in tumours for long periods before they become rapidly growing carcinomata, and it would therefore appear that all these tumours are carcinomatous from the start. The distribution of the carcinomatous tumours would indicate that the whole of this substrain is cancerous, and that this condition was attained in the tumour in Rat 5, Passage 5A, which supplied the grafts for Passage 6F. From the proliferative characters of this tumour it appears likely that during its two years' growth in a female rat it was stimulated in some way, and thus attained its capacity to show malignant properties in later passages.

At whatever stage this substrain acquired its apparently new properties, it is evident that a considerable interval may elapse between the attainment of malignancy and the appearance of the cellular and invasive characters of malignancy. It appears likely that during this latent period the actively growing and abundant fibrous component may have a restraining and formative influence on the cancerous epithelium. At a later stage, when the epithelium is more active and appears carcinomatous, the fibrous tissue is frequently much reduced and infiltrated by inflammatory cells. This apparently defensive reaction of the host is well shown in Fig. 10, where the carcinomatous tissue has called forth a heavy cellular reaction which is absent in the fibroadenomatous part of the tumour. This reaction may be a response to new antigenic properties in the carcinomatous tissue, or it may be called forth by some product of the infiltrative action of the cancerous epithelium. Whatever the stimulus may be the reaction appears capable of exerting a restraining influence on the growth of carcinoma, and in one slow-growing tumour, in Rat 5, Passage 8D (Fig. 2), an eventual complete regression was effected apparently by the very heavy inflammatory reaction.

Further observations on this cancerous substrain may help to elucidate the nature of the change which occurred in the tumour in Rat 5, Passage 5A, during its long sojourn in a female environment. Against the change being an example of carcinoma arising spontaneously in a benign tumour as in normal breast are the failure to find carcinoma in this tumour, and the long intervals, 27 to at least 63 weeks, before carcinoma became manifest in passage tumours. On the other hand, this rat fibroadenoma may be regarded as a

mixed tumour in which the fibrous and glandular components in their normal relationships exercise a restraining and organizing influence on each either. It has already been suggested that the fibrous component has intrinsic malignant properties, and that the appearance of fibromata and sarcomata in old fibroadenomata is due to a release from the restraining influence of the epithelium (Selbie, 1941). In a similar way the cancerous nature of this substrain can be ascribed to a greater proliferative capacity in the glandular component acquired by prolonged stimulation in a female environment. By virtue of its greater capacity for growth the epithelium can outstrip the fibrous tissue and thus, by overcoming the restraining influence of the fibrous component, make manifest its intrinsic malignant properties.

SUMMARY.

A cancerous substrain of a transplantable rat fibroadenoma is described.

In this substrain five tumours in three successive passages have become manifestly carcinomatous at long intervals after inoculation.

It is suggested that these changes can be ascribed to an increased proliferative capacity of the glandular tissue acquired by the parent tumour of the substrain. By its increased powers of proliferation the epithelium can overcome the restraint of the fibrous component of the tumour and can thus make manifest its intrinsic malignancy.

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THE INFLUENCE OF CHEMICAL CONSTITUTION ON ANTISEPTIC ACTIVITY. I: A STUDY OF THE MONO-AMINOACRIDINES.

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THE di-aminoacridines ("flavines") are a well-known group of antiseptics which strongly inhibit bacteria in the presence of tissue fluids and are relatively innocuous to polymorphonuclear leucocytes. The antiseptic action of this group was discovered by Browning (1913), who introduced two members, acriflavine and proflavine, into clinical use. It is noteworthy that what little is known of the relationship between chemical constitution and antiseptic action in the acridine series has been revealed mainly through the researches of British workers. First of all, Browning, Cohen, Gaunt and Gulbransen (1922) proved that antiseptic activity was due to the complete acridine nucleus and was not manifested by simpler analogues derived from pyridine and quinoline. Furthermore, they demonstrated that maximum antiseptic action was developed only when amino substituents were present in the acridine nucleus. Later Albert, Francis, Garrod and Linnell (1938) investigated the biological activity of a series of ten di-aminoacridines, and showed that the effect of the compounds could be correlated, in a general way, with the positions occupied by the amino groups in the acridine molecule. Simultaneously Albert, Dyer and Linnell (1937) examined the same compounds for toxicity in mammals.

The present investigation, a logical extension of the researches already described, has been undertaken to achieve something more fundamental regarding the relationship between structure and action in the acridine antiseptics. Up to the present, attention has been focused on the di-aminoacridines and their more complex derivatives, such as rivanol (2 : 5 diamino-7-ethoxyacridine), whilst the simpler mono-aminoacridines have passed almost unnoticed, doubtless because of their relative inaccessibility. Nevertheless, it is expected that in these simpler mono-amines the correlations sought after would be more readily demonstrated and interpreted than has so far been found possible for the di-amines. New methods for the preparation of the mono-aminoacridines (of which the five possible isomerides have been known since 1938) were therefore worked out (Albert and Ritchie, 1941) and provided the material for these investigations.

CHEMISTRY OF THE MONO-AMINOACRIDINES.

In general, 2-chlorobenzoic acid was condensed with aniline, with *o*-, *m*-, and *p*-nitroanilines, and with *m*-phenylenediamine (for the 5-, 1-, 4-, 3- and 2-isomerides respectively). The resultant diphenylamine-2-carboxylic acids were cyclized with sulphuric acid or phosphorus oxychloride and, in the cases of the 1-, 3-, and 4-nitroacridones and the 2-aminoacridone so obtained, reduction was carried out with aluminium amalgam giving the corresponding amino-acridanes which were oxidized by ferric chloride to the required aminoacridines. The amino group of 5-aminoacridine was inserted by heating with ammonium carbonate.

It will be seen (Table I below) that the five isomeric monoaminoacridines differ widely in physical properties. The range of dissociation constants in water is of particular interest, since it is too extensive to be attributed to mere positional isomerism, and consequently it becomes necessary to seek evidence of structural alteration in the molecule, namely, changes in the disposition of bonds.

It is evident that 3- and 4-aminoacridines are normal amino derivatives of acridine (in the sense that aniline is a normal amino derivative of benzene), since the addition of a $-NH_2$ group to an aromatic ring has caused a small but definite increase in basicity over and above that contributed by the acridine nucleus.

In the case of 1-aminoacridine, on the other hand, the addition of an amino group has lowered the basicity to one half that of the parent nucleus acridine. This is reminiscent of the behaviour of other *ortho* di-amines, particularly 1 : 2 di-aminobenzene, shown by Farmer and Warth (1904) to be less basic than aniline. In spite of the relative weakness of the N—H—N linkage, it seems permissible to assume that we are dealing with divalent hydrogen and that, in the case of 1-aminoacridine, the usual structure has been replaced by a hydrogen-bonded form (illustrations 1 and 1A respectively). Further evidence in favour of this hydrogen bonded form is afforded by the relatively greater tendency of 1-aminoacridine to pass from water to oil compared with the behaviour of the other isomerides (Table I, oil/water partition coefficients).

The case of the 2- and 5-aminoacridines is quite different, in that the addition of an amino group to the acridine nucleus is followed by an enormous increase in basicity of the product. Such an increase of one hundred thousand fold can only be explained by an isomeric change of the amino ($-NH_2$) into an imino ($=NH$) form, such as occurs in crystal violet and brilliant green. Several cases of increased basicity depending on amino-imino tautomerism have been described by Lecher and Graf (1924–5), whilst Sidgwick (1937) showed that this phenomenon occurred whenever the resulting ion would increase the resonance of the molecule.

In view of the above evidence the formulae of the isomeric mono-amino-acridines are depicted in Fig. 1.

Formula IA represents the hydrogen bonded form of 1-aminoacridine, in which the ring nitrogen being completely blocked is prevented from forming salts; IIA the imino form of 2-aminoacridine (from which its salts are derived);

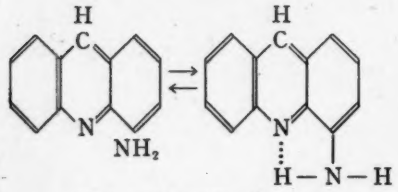
TABLE I.—*Physical Properties of Substances Used.*

Substance.	Melting-point of free base.	Strength of base* Dissociation constant in water at 25° C.	Partition† coefficient oil/water.	Colour of solid.		Fluorescence in solution.		Solubility of hydrochloride in cold water per cent.	Solubility of hydrochloride in 0·85% saline per cent.
				Base.	Salt.	Base.	Salt.		
5-Aminoacridine	233° C.	$300{,}000 \times 10^{-10}$	1	Yellow	Cream	Green	Blue	0·5	0·05
2 : 8 Di-aminoacridine (proflavine)	277° C.	$120{,}000 \times 10^{-10}$	0·5	Brown	Red	,,	Green	1·0	0·3
2-Aminoacridine	217° C.	$12{,}000 \times 10^{-10}$	5	Yellow	Scarlet	,,	,,	0·5	0·1
4-Aminoacridine	180° C.	30×10^{-10}	55	Scarlet	Violet	Pale orange	None	1·0	1·0
3-Aminoacridine	213° C.	12×10^{-10}	90	Yellow	Brown	Green	,,	0·2	0·2
Acridine	110° C.	2×10^{-10}	250	White	Cream	Blue	Green	Hydrolysed	Hydrolysed
1-Aminoacridine	108° C.	1×10^{-10}	1200	Brown	Red-brown	None	None	,,	,,

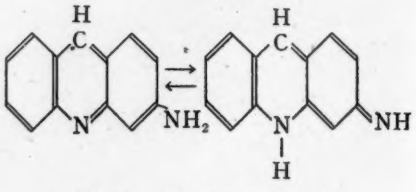
* Albert, Rubbo and Goldacre (1941).
† Albert, Goldacre and Rubbo (1941).

III and IV, the normal amines, 3- and 4-aminoacridines respectively; and VA the imino form of 5-aminoacridine.

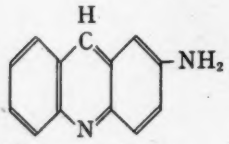
This physical study of structural isomerism is being supplemented by chemical and spectrographic determinations. In the next section the profound influence of these structural variations on biological properties is described.



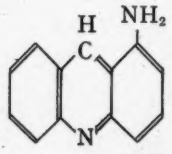
(I) 1-Aminoacridine (IA).



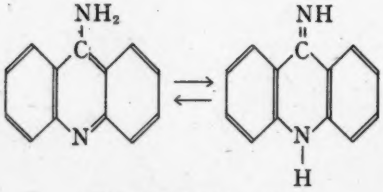
(II) 2-Aminoacridine (IIA).



(III) 3-Aminoacridine.



(IV) 4-Aminoacridine.



(V) 5-Aminoacridine (VA).

FIG. 1.

BIOLOGICAL TESTS.

In order to assess the influence of chemical constitution on biological activity three widely separate systems were selected for study, namely, bacterial, mammalian and leucocytic systems. In this manner it has been possible to compare the lethal action of each member of this isomeric series towards diverse types of living cells, and to assess the value of each substance as a possible clinical antiseptic. It will be shown that an orderly sequence in regard to increasing antiseptic activity and increasing toxicity for mice is achieved for the seven substances tested and that this same order is maintained when they are grouped according to increasing degrees of basicity, as determined by

potentiometric titrations (Albert, Rubbo and Goldacre, 1941). Moreover, as antiseptic action and toxicity increases, the tendency of the substance to pass from oil to water markedly decreases (Albert, Goldacre and Rubbo, 1941). These remarkable correlations will be discussed later. Of immediate practical importance has been the discovery of 5-aminoacridine whose *in vitro* action is, in some respects, superior to proflavine, whilst, in addition, the substance possesses desirable features of being non-staining and non-irritating. As our standard for comparison of antiseptic activity we have used proflavine, a substance of proved clinical usefulness, and not acriflavine, which, being a variable mixture of proflavine and its methochloride (Gailliot, 1934), is variable in activity and consequently unsuitable. In some of the tests, acridine, the progenitor of this series, was also included.

ACTION ON BACTERIA.

A. *Bacteriostatic Tests.*

The technique adopted in this investigation has been based on the method described by Albert, Francis, Garrod and Linnell (1938), the only departure being in the selection of test organisms. The cultures included were as follows: *Streptococcus pyogenes* (Group A) isolated from a case of puerperal sepsis, *Staphylococcus aureus* (coagulase positive) from a human infection, *Clostridium welchii* (capsulated strain) from a case of gas gangrene, whilst *Bacterium coli* and *Proteus vulgaris* were old laboratory cultures. All tests were performed in duplicate.

TABLE II.—*Highest Dilutions of Antiseptic Completely Preventing Visible Growth in* 48 *Hours at* 37° *C.*

Substance.	Medium : Broth pH 7·4.					Medium : 10% serum broth pH 7·2.					Bacteriostatic index. (Sum-total of inhibitory dilutions.)
	Cl. welchii.	*Strep. pyogenes.*	*Staph. aureus.*	*B. coli.*	*Proteus vulgaris.*	*Cl. welchii.*	*Strep. pyogenes.*	*Staph. aureus.*	*B. coli.*	*Proteus vulgaris.*	
5-Aminoacridine	6	5	3	3	3	7	6	3	3	4	43
2 : 8 Di-aminoacridine (proflavine)	6	6	3	3	2	7	6	3	3	2	41
2-Aminoacridine	6	5	2	4	3	7	4	3	4	3	41
4-Aminoacridine	4	3	0	1	0	5	2	1	1	0	17
3-Aminoacridine	4	3	1	1	0	5	2	1	0	0	17
Acridine	3	2	1	0	0	3	1	1	0	1	12
1-Aminoacridine	3	1	0	0	0	2	1	1	0	0	8

Key to dilutions :

0 = Growth in all dilutions.
1 = 1 in 5,000 of antiseptic in the medium.
2 = 1 in 10,000 " " "
3 = 1 in 20,000 " " "
4 = 1 in 40,000 " " "
5 = 1 in 80,000 " " "
6 = 1 in 160,000 " " "
7 = 1 in 320,000 " " "
8 = 1 in 640,000 " " "

On examining the results (Table II) it is apparent that the Gram-positive organisms are more susceptible to the acridine antiseptics than are the Gram-negative types. Of the cultures used *Cl. welchii* was the most susceptible to each compound, whilst next in order of susceptibility was *Strep. pyogenes*, followed by *Staph. aureus*, *B. coli*, and finally *Proteus vulgaris*. 5-Amino-acridine appears to be slightly superior to proflavine as a bacteriostatic agent, the difference being due only to its greater inhibitory effect on *Proteus vulgaris*.

B. *Bactericidal Tests.*

The choice of a method for determining bactericidal activity is generally open to criticism. It is now agreed that if blood is used as a suspending fluid it is essential that it be made to " circulate " during the time of the experiment, otherwise quite erroneous results may be expected. In all the present work a system closely imitating blood in circulation was provided for by means of the rotating-tube machine, illustrated below.

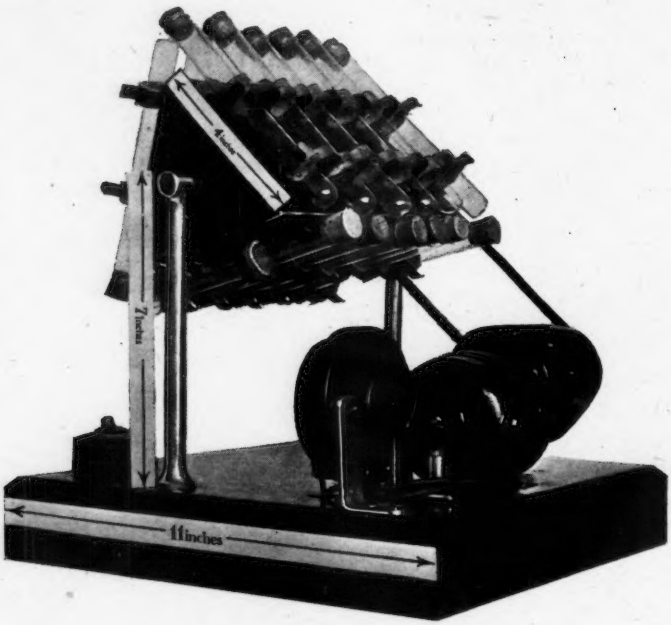


FIG. 2.

Using this apparatus the bactericidal capacity of each compound was determined in both an artificial (serum broth) and in what may be termed a " living blood " environment.

The culture *Strep. pyogenes* (Group A) used in the bacteriostatic tests was selected. In order to eliminate error due to chain disruption, an 18-hour

serum broth culture of the organism was first vigorously shaken for 5 minutes by hand. The shaken culture was centrifuged and washed in saline to remove soluble haemolysins, and finally resuspended in saline for use in the test proper.

Suspending fluids.—(1) Serum broth, i.e. nutrient broth containing 10 per cent. of ox serum.

(2) " Living blood " (for the *vivo*-in-*vitro* test) was prepared by bleeding a sheep from the jugular vein, collecting the blood aseptically, citrating to give a final concentration of 2 per cent. sodium citrate, and maintaining at 37° C. before use. This blood was used within half an hour of bleeding.

The serum of the sheep used as our reservoir of blood had been previously tested for the presence of agglutinins and precipitins acting on the *Strep. pyogenes* with negative results.

Test substances.—These were dissolved in sterile distilled water acidified with HCl calculated to form the monohydrochloride of the base. The final strength of the solutions in each case was 0·2 per cent. of base.

Test proper.—1·9 ml. of suspending fluid was measured by pipette into sterile Wassermann tubes, and then 0·1 ml. of test substance and 0·05 ml. of bacterial suspension were added in this order. Thus the final concentration of antiseptic was 1 in 10,000. The tubes were corked, inserted into the machine and rotated at a rate of 14 inversions per minute for two hours at 37° C. for the *vivo*-in-*vitro* test, and for one hour in the case of serum broth. In the *vivo*-in-*vitro* test the tubes and corks were paraffined to prevent adhesion of leucocytes to the walls of the tube and each test was set up in a 37° C. incubator room. Zero and final counts were determined by pipetting 1 ml. quantities of appropriate dilutions of the suspensions into melted blood agar plates and counting the number of colonies developing after 48 hours' incubation at 37° C. For each counting the mean count of three plates was taken.

The pH of the different suspending fluids containing the antiseptic lay between 7·0–7·4, as shown by measurements made with a glass electrode.

The disinfection coefficient, K, was calculated according to the equation of a reaction of the first order, viz. :

$K = \frac{1}{T} \log \frac{a}{a-x}$, where T = time in hours, a = a number of organisms at the beginning and x = number of organisms killed. Experiments, which need not be detailed here, showed that K values determined at half-hourly intervals were fairly constant except during the first half hour of disinfection, when they were invariably high.

RESULTS.

The results are given in Tables III and IV below.

Dealing first with the bactericidal action in serum broth (Table III), it will be seen that 5-aminoacridine was the most active of the aminoacridines, and slightly more effective than proflavine. It will be noted that the order of activity in bactericidal power coincides with the order determined in the bacteriostatic experiments (Table II).

The most striking feature of the *vivo*-in-*vitro* test, Table IV, was the marked reduction in disinfection rate when compared with that found in serum broth.

TABLE III.—*The Bactericidal Action at* 1 *in* 10,000 *Concentration of Antiseptic on* Streptococcus pyogenes *in the Presence of* 10 *per cent. Serum Broth.*

Substance.	Experiment number.	Zero.	1 hour.	Percentage of organisms killed in 1 hour.		Mean $K^{s.b.}$
5-Aminoacridine	1	3,525,000	100,000	97		
	2	4,875,000	150,000	97	96	1·39
	3	9,875,000	500,000	95		
2 : 8 Di-aminoacridine (proflavine)	1	4,600,000	450,000	90		
	2	6,000,000	475,000	92	92	1·14
	3	10,400,000	700,000	93		
2-Aminoacridine	1	2,350,000	600,000	75		
	2	5,500,000	1,400,000	74	82	0·84
	3	7,500,000	225,000	97		
4-Aminoacridine	1	3,300,000	1,325,000	60		
	2	7,600,000	3,050,000	60	61	0·42
	3	7,850,000	2,825,000	64		
3-Aminoacridine	1	3,750,000	1,650,000	56		
	2	4,075,000	1,750,000	57	56	0·36
	3	6,425,000	2,850,000	56		
1-Aminoacridine	1	5,175,000	2,625,000	49		
	2	5,750,000	2,900,000	48	49	0·30
	3	8,875,000	4,450,000	50		
Control : Without antiseptic	1	3,250,000	3,900,000	Increase 20%		
	2	5,450,000	6,750,000	,, 24%	20%	
	3	7,300,000	8,550,000	,, 17%		

TABLE IV.—*The Bactericidal Action at* 1 *in* 10,000 *Concentration of Antiseptic on* Streptococcus pyogenes *in the Presence of "Living Blood"* = (Vivo-*in*-vitro *Test*).

Substance.	Experiment number.	Zero.	2 hours.	Percentage of organisms killed in 2 hours.		Mean $K^{v.v.}$
5-Aminoacridine	1	2,925,000	275,000	91		
	2	4,225,000	325,000	92	93	0·58
	3	7,250,000	400,000	95		
	4	10,875,000	725,000	93		
2 : 8 Di-aminoacridine (proflavine)	1	2,200,000	300,000	86		
	2	2,650,000	450,000	83	84	0·40
	3	4,100,000	700,000	83		
	4	7,125,000	1,050,000	85		
2-Aminoacridine	1	2,175,000	975,000	55		
	2	2,500,000	1,250,000	48	51	0·15
	3	3,250,000	1,625,000	50		
	4	6,575,000	3,175,000	52		
4-Aminoacridine	1	2,325,000	925,000	61		
	2	3,550,000	1,625,000	54	55	0·17
	3	7,200,000	3,250,000	55		
	4	9,150,000	4,275,000	53		
3-Aminoacridine	1	1,550,000	1,350,000	13		
	2	2,900,000	2,550,000	30	17	0·04
	3	6,425,000	5,350,000	17		
	4	6,625,000	5,525,000	17		
1-Aminoacridine	1	2,075,000	1,800,000	13		
	2	3,350,000	2,825,000	16	15	0·04
	3	6,350,000	5,275,000	17		
	4	9,350,000	8,150,000	13		
Control : without antiseptic	1	1,350,000	1,900,000	Increase 40%		
	2	3,075,000	4,375,000	,, 31%	35%	
	3	3,350,000	4,650,000	,, 34%		
	4	4,700,000	6,350,000	,, 35%		

At least two hours' exposure in blood was needed to effect a reduction in the number of streptococci in this medium comparable to the reduction effected after one hour's exposure in serum broth. The two sets of disinfection coefficients are compared in Table V.

TABLE V.—*Bactericidal Action.*

Substance.	Disinfection in serum broth $K^{s.b.}$	Coefficient in "living blood" $K^{v.v.}$	Percentage reduction in bactericidal activity due to whole blood.
5-Aminoacridine	1·39	0·58	58%
2 : 8 Di-aminoacridine (proflavine)	1·14	0·40	65%
2-Aminoacridine	0·84	0·15	81%
4-Aminoacridine	0·42	0·17	60%
3-Aminoacridine	0·36	0·04	88%
1-Aminoacridine	0·30	0·04	86%

$$\frac{(K^{s.b.} - K^{v.v.})}{K^{s.b.}} \times 100 = \% \text{ reduction due to blood.}$$

It is evident that bactericidal power of all substances examined is greatly reduced by blood and that the extent of this reduction varies over a wide range, viz. from 58 per cent. reduction with 5-aminoacridine to 88 per cent. reduction with the 1- and 3-aminoacridines.

TOXICITY FOR MICE.

The method chosen for the toxicological examination was identical with that described by Albert, Dyer and Linnell (1937). The number of mice killed after 3 days was noted and the average lethal dose, L.D.50 (the dose which causes death in 50 per cent. of the group of mice), was determined. The L.D.50 dose was calculated in this work by plotting the logarithm of the dose against percentage mortality. The curve so constructed was in each experiment a straight line composed of at least four readings. From these curves the L.D.50 doses were read directly.

The immediate physical response of the mice injected with 4- and 5-aminoacridines differed strikingly from that with other isomerides. The 4-aminoacridine produced slight tetanic spasms followed by coma about forty minutes after injection. This effect was noted by Albert, Dyer and Linnell (1937). The 5-aminoacridine, however, caused marked tetanic spasms affecting mainly the hind quarters ten minutes after injection, and death generally followed within thirty minutes with a generalized tetany and with the protrusion of the eyeballs. The action of 1-aminoacridine was manifested 24 hours after injection as an extensive necrosis of the skin around the site of injection. This necrotic lesion was severe, the entire skin, including subcutaneous tissue and superficial fascia, entirely disappearing and exposing extensive areas of muscle. Probably because of this violent local necrosis the substance is not disseminated throughout the body and consequently is relatively non-toxic. The mice injected with 2- and 3-aminoacridine generally died 18 hours after injection without showing any marked symptoms.

TABLE VI.—*Comparison of Toxicities by Subcutaneous Injection of Mice. Results Expressed as Average Lethal Dose of Free Base.*

Substance.	Dose g. per kg. body weight.	Strength of solution.	Number of mice injected.	Number of mice killed after 24, 48, 72 hours.	L.D. 50 in g. per kg. of body weight.	L.D. 50,* Albert, Dyer and Linnell (1937).
5-Aminoacridine	0·03	1 : 400	6	0 0 0		
	0·05	,,	18	4 4 4	0·08	—
	0·10	1 : 200	12	8 8 8		
	0·15	,,	6	6 6 6		
2 : 8 Di-aminoacridine (proflavine)	0·05	1 : 400	6	0 0 0		
	0·10	1 : 200	6	2 2 2		
	0·15	,,	6	3 3 3	0·14	0·15
	0·20	,,	12	8 9 10		
	0·30	,,	6	6 6 6		
2-Aminoacridine	0·10	1 : 200	6	0 0 0	0·17	0·30; since corrected to to 0·17 (see text).
	0·15	,,	12	3 3 3		
	0·20	,,	12	9 9 9		
	0·30	,,	12	12 12 12		
3-Aminoacridine	0·20	1 : 200	6	0 0 0		
	0·30	,,	6	2 2 2	0·33	—
	0·40	,,	6	4 4 4		
	0·50	,,	6	5 6 6		
4-Aminoacridine	0·20	1 : 200	6	0 0 0		
	0·30	,,	12	3 4 5	0·33	0·25
	0·40	,,	6	4 4 4		
	0·50	,,	6	6 6 6		
1-Aminoacridine	0·70	1 : 100	6	0 0 0		
	0·80	,,	6	1 2 2	0·91	—
	0·90	,,	6	1 2 2		
	1·00	,,	6	3 4 4		
Acridine	—	—	—	— — —	—	0·30

* Recalculated as free base.

In Table VI we have included some L.D.50 results obtained by Albert, Dyer and Linnell (1937), and it will be noticed that the figure for 2-aminoacridine is at variance with our findings. However, Dyer (private communication, 1941), using a larger number of mice, has since obtained our figure for the L.D.50 of this substance and an apparent discrepancy is thus removed.

TOXICITY TO HUMAN POLYMORPHONUCLEAR LEUCOCYTES.

This property was determined by Wright's technique (Wright and Colebrook, 1921), in which the reduction in phagocytosis in the presence of antiseptic, as compared with a saline control, was measured. The extent of this reduction in phagocytic activity was interpreted as being a fair index of toxicity to polymorphonuclear leucocytes.

Experimental.

Equal volumes (approx. 0·03 ml.) of fresh human blood, sodium citrate, bacterial suspension (18 hour *Strep. pyogenes* culture, washed, resuspended in saline), and saline solution of the acridine mono-hydrochloride under test, were drawn up into a Pasteur pipette, mixed by blowing out several times on to a paraffined slide, sealed in the flame, and incubated horizontally at 37° C. for one hour. Controls with saline replacing the acridine were put up with every test. All tests were done at least in triplicate. At the end of the incubation period the blood mixture from each pipette was blown out on to four glass slides, and films were made by using a slightly concave spreader. After fixing, and staining with dilute carbol thionin, phagocytic counts were performed in which not less than 20 leucocytes per slide were counted.

TABLE VII.—*Phagocytosis of* Streptococcus pyogenes *by Polymorphonuclear Leucocytes in the Presence of Acridine Compounds.*

Substance.	Percentage reduction in phagocytosis in presence of—			
	1/1000.	1/2000.	1/4000.	1/10,000.
2-Aminoacridine	22	14	12	3
2 : 8 Di-aminoacridine (proflavine)	47	36	30	26
4-Aminoacridine	50	46	35	27
3-Aminoacridine	50	45	37	28
5-Aminoacridine	53	49	37	44
1-Aminoacridine	87	78	73	54

It will be seen that in a concentration of 1 in 1000 there is no significant difference between the derivatives except that 2- and 1-aminoacridines represent extremes, the former being almost innocuous to the phagocytic cells, whilst the latter is extremely toxic. With dilution a progressive decrease in toxicity is generally to be noted throughout the series, but an exception to this is seen in the case of 5-aminoacridine, whose toxicity is not greatly reduced on dilution.

DISCUSSION.

The starting-point for a discussion on the connection between structure and action in this series is found in a piece of work that initiated the present studies (Albert, Francis, Garrod and Linnell, 1938). The general conclusions drawn by these authors were : (1) Acridines containing an amino group in the 1-position were devoid of antiseptic activity even though a strongly acting antiseptic substituent such as an amino group in the 2-position was also present ; (2) acridines containing an amino group in the 3- or 4-position had moderate antiseptic activity ; (3) for outstanding antiseptic activity an amino group was required in the 2- or 5-position. These conclusions, which were deduced on theoretical grounds from a study of di-aminoacridines, have been experimentally confirmed in the present work by employing acridines containing only one amino group. In the light of physico-chemical data made available this year, it has become possible to put forward some tentative explanations of this relationship of positional isomerism to antiseptic action.

TABLE VIII.—*Summary of Physical and Biological Properties of the Mono-aminoacridines and Some Related Substances.*

Substance.	Strength of base (dissociation constant in water, 25° C.) from Table I.	Partition co-efficient oil/water from Table I.	Bacteriostatic index from Table II.	Disinfection co-efficient. 1 in 10,000 concentration in—		Percentage reduction in bactericidal activity due to whole blood from Table V.	Average lethal dose for mice in g. per kg. of body weight from Table VI.	Percentage reduction in phagocytosis by 1/10,000 concentration from Table VII.
				Serum broth from Table III.	Whole blood from Table IV.			
5-Aminoacridine	$300{,}000 \times 10^{-10}$	1·0	43	1·39	0·58	43	0·08	44
2 : 8 Di-aminoacridine (proflavine)	$120{,}000 \times 10^{-10}$	0·5	41	1·14	0·40	65	0·14	26
2-Aminoacridine	$12{,}000 \times 10^{-10}$	5	41	0·84	0·15	82	0·17	3
4-Aminoacridine	30×10^{-10}	55	17	0·42	0·17	60	0·33	27
3-Aminoacridine	12×10^{-10}	90	17	0·36	0·04	89	0·33	28
Acridine	2×10^{-10}	250	12	—	—	—	0·30	—
1-Aminoacridine	1×10^{-10}	1200	8	0·30	0·04	92	0·91	54

— = Experiment not done.

In Table VIII our quantitative results have been assembled, and it becomes evident that the antiseptic action shows a very remarkable correlation with the basicity of the compound. It will be seen that when the mono-aminoacridines are arranged in order of increasing bacteriostatic activity, of increasing bactericidal power in serum broth, and of increasing toxicity, a uniform sequence is maintained, and that this order of biological activity corresponds to an order of increasing chemical basicity. Moreover, proflavine (2 : 8 diaminoacridine) and acridine itself occupy positions in the biological order corresponding to their degrees of basicity. From this correlation of biological activity and basicity the question arises as to whether it is the result of a purely chance phenomenon or of something more fundamental. From a consideration of seven compounds only it would be premature to come to any final decision, but on the evidence so far accumulated two interpretations are put forward.

The first interpretation is that a high basicity, as possessed by proflavine and 5-aminoacridine, appears to be an essential attribute for marked antiseptic activity in this series, and simply facilitates the union between the drug and the acid group of the bacterial protein in the manner suggested for crystal violet by Stearn and Stearn (1924). In this respect it is interesting to note that Gram-positive organisms, which have greater affinity for basic dyes than have the Gram-negative types, are particularly susceptible to proflavine, which is a typical basic dye.

The other interpretation is that the more active drugs in this series owe their increased antiseptic action to the presence of the peculiar imino structure (see Fig. 1, Formulae IIA and VA) which arise by tautomerism of the 2- and 5-amino derivatives of acridine. These forms would probably not, in themselves, be important did they not lead to resonance between the ionic forms, e.g. in the case of 2-aminoacridine.

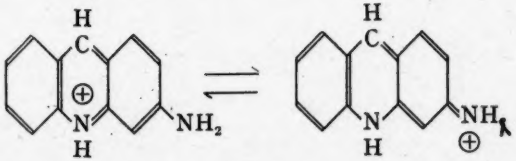


FIG. 3.

As was pointed out by Sidgwick (1937), such resonance brings the ions to a lower energy level than the free bases and hence such substances acquire an extra impetus to salt-formation, this tendency being the actual cause of their high basic strength.

This grouping (= NH), and its resonance effects, is established as fundamental to the structure of another class of antiseptic dyes, the poly-phenylmethanes (brilliant green, " gentian violet," etc.), so that its recognition in the more effective members of the acridine series now brings the latter into line with the above dyes. If, then, the antiseptic activity of acridine compounds is dependent on the state of combination of the nitrogen atoms present, a tentative classification may be made along the following lines : A resonating

imino structure confers marked antiseptic action (2- and 5-aminoacridines); a normal amino structure confers moderate antiseptic action (3- and 4-aminoacridines); whilst the hydrogen bonded amine evinces no significant activity (1-aminoacridine). These suggestions imply that basicity is significant only as an index of the reactive condition of the nitrogen atoms and is not, *per se*, the determining factor in the biological activity of the compounds.

As a general working hypothesis of the theory of toxicity in the acridine series it is tentatively suggested that the ring nitrogen is the toxic influence in the intact acridine nucleus. The addition of an amino group to the ring does not increase the toxicity of the new compound, unless a resonating imino form is produced, as in 2- and 5-aminoacridines, in which case the toxicity is increased. Again, when the ring nitrogen is masked by the new substituent, as in 1-aminoacridine, the toxicity is lowered. This theory is upheld for proflavine, which may be considered to contain one imino group and one true amino group. However, this hypothesis must be treated as tentative until determinations have been performed on di-aminoacridines (additional to those of Albert, Dyer and Linnell (1937)).

No matter what further investigations may contribute to our knowledge of this relationship, it can already be laid down that proflavine and 5-aminoacridine represent as high a degree of basicity as is necessary for the display of marked antisepsis. This may be deduced from the evidence that compounds of yet higher basicity, such as acridine orange and acriflavine, the latter having a basicity approaching that of the caustic alkalis, are in no way superior as antiseptics.

A further correlation of physical and biological properties in this series has recently come to light (Albert, Goldacre and Rubbo, 1941). If the seven compounds are arranged in the order of increasing antiseptic strength, etc. (Table VIII), they are found to be in the order of their increasing readiness to pass from oil into water. The significance of this phenomenon and that of the basicity relationship discussed above is being sought in a much larger group of aminoacridines so selected as to test the validity of these relationships.

So far it has proved impossible to reduce the two remaining biological properties detailed in Table VIII (action on phagocytes; reduction of disinfectant action by whole blood) to a chemical basis. It may well be that such correlations will become apparent when other physical properties are determined for this series, such as oxidation-reduction potential and adsorption at oil— and protein— interfaces. Whilst it is a matter of practical experience that the more basic of the aminoacridines are the most difficult to reduce, the exact measurement of the redox potentials has proved difficult because of the poor solubility of the free bases in water, and the ease with which they are precipitated by the common buffers and reagents. Less difficulty, however, is anticipated in obtaining data relevant to the surface activities of these compounds.

One discovery of practical value emerging from the present work is that 5-aminoacridine, which has the clinically desirable properties of proflavine, does not stain the skin. It would seem that there is a distinct sphere of usefulness for this substance in dermatology, in ophthalmology, and in ear, nose and throat work. The preliminary reports from some Victorian hospitals

that have co-operated in testing this substance clinically have been distinctly encouraging.

SUMMARY.

1. The physical and chemical properties of the five mono-aminoacridines have been described.

2. These compounds have been examined for their bacteriostatic and bactericidal powers, their toxicity for mammalian tissue and their effect on phagocytic action. Proflavine (2 : 8 di-aminoacridine) has been included in these experiments for purposes of comparison.

3. It has been found that biological activity in this series runs closely parallel to chemical basicity which is attributed to variation in the state of chemical union of the nitrogen atoms in the acridine nucleus.

4. It has been tentatively suggested that in the present series three classes of substances are to be distinguished, namely, Class 1 with marked antiseptic action associated with a relatively high chemical basicity and the imino (= NH) grouping ; Class 2 with moderate antiseptic action, moderate basicity and the normal amino (— NH_2) grouping, and Class 3 with little or no antiseptic action and possessing an abnormally low basicity associated with an amino group that is masked through hydrogen bonding (— $NH_2 \ldots N$). Proflavine, 2- and 5-aminoacridines have been assigned to Class 1 ; 3- and 4-aminoacridines to Class 2 ; and 1-aminoacridine to Class 3.

5. The toxicity of these compounds for mice has been found to place these compounds in the same relative order as do the antiseptic values, but the toxicity for leucocytes follows another sequence.

6. 5-aminoacridine is recommended for clinical trial as a non-staining antiseptic possessing all the valuable properties of proflavine.

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ARTIFICIAL ANTIGENS WITH AGAR; GUM ACACIA AND CHERRY GUM SPECIFICITY.

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THE conversion of a bacterial polysaccharide into an antigenic complex that will induce the formation of immune-body complex specific for the polysaccharide component of the complex has been reported in earlier papers (Morgan and Partridge, 1940*a*, 1941*a*, *b*; Partridge and Morgan, 1940). The method of combining bacterial polysaccharide and protein components in formamide solution has now been extended to include the formation of antigenic complexes that contain as the serological specific haptens polysaccharides of vegetable origin, and in the present paper the conversion of agar-agar, gum acacia and cherry-gum into antigenic complexes is described. A preliminary account of the work with the artificial agar antigen has already appeared (Morgan and Partridge, 1940*b*).

EXPERIMENTAL.

The experiments were made with the polysaccharides, agar-agar, kanten, gum acacia and cherry gum. In each instance the commercial material was subjected to a simple purification procedure that removed the gross impurities present and reduced considerably the nitrogen content of the polysaccharide preparations.

The agar antigenic complex.

A commercial sample of agar was freed from gross impurities and inorganic matter by solution in anhydrous formamide. The clear viscous solution was fractionated by precipitation with alcohol, and the main fraction that separated between the alcohol levels 50 per cent. and 60 per cent. was again dissolved in formamide. The fraction was precipitated from solution by 60 per cent. alcohol, and thoroughly washed with 60 per cent. alcoholic formamide and finally with absolute alcohol. The agar was then dissolved in boiling water to make a 0·1 per cent. solution and after cooling, dialysed for several days against distilled water at 0°. The purified agar was recovered by the addition of alcohol to the dialysed solution, the material being subsequently washed with alcohol and dried *in vacuo*. The substance contained 0·8 per cent. N and was tested

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for antigenicity in rabbits (Table I, animals 140, 141, 170 and 171). Two animals received eight doses of 0·05 mg. and two others ten doses of 0·2 mg., but in no instance were agar precipitins subsequently detected in the sera of these animals. For convenience, kanten, a breakdown product of agar, was used as hapten in the precipitation tests.

A portion of the agar (60 mg.) was dissolved in 3 ml. of anhydrous formamide (m.p. + 2°) and a sample of conjugated protein (40 mg.) prepared from the specific somatic antigen of *Bact. shigae* was added as a dry powder. The conjugated protein was prepared according to the method described by Morgan and Partridge (1940*a*, 1941*a*, *b*) and contained 11·4 per cent. N. The protein slowly passed into solution. The mixture was then allowed to stand at 37° for 2 hours, and, after increasing the volume by the addition of 10 ml. of formamide, was kept at 0–2° for 48 hours. The agar-protein complex was recovered by the addition of alcohol to the formamide solution, the precipitate was thoroughly washed with alcohol, was taken up in 10 ml. of boiling water and, after cooling, was evaporated to dryness from the frozen state. Samples of the material (10 mg.) were prepared for animal inoculation by suspension in 10 ml. of sterile saline and boiling for a few seconds to ensure solution or fine dispersion. The opalescent, colloidal solution that was formed, however, was unstable on cooling, and a sediment of fine particles appeared on standing at room-temperature for several hours.

The whole suspension was given to rabbits Nos. 142 and 143 (Table I). Three doses each of 0·05 mg. failed to induce the formation of specific precipitins, but after a rest period of one week and a further course of five 0·2 mg. doses, the serum of both animals gave precipitation when mixed with an equal volume of 1 in 10^4 or 10^5 dilution of kanten. Definite, though slight, precipitation was also given by the kanten preparation at a dilution of 1 in 10^6.

The gelatinous character of a solution of the antigenic complex suggested that the preparation contained an excess of uncombined agar. The material was therefore dissolved in boiling water, allowed to cool slowly and to stand for some hours until the formation of the sparingly soluble floccules was complete. The suspension was then centrifuged and the deposit washed with small quantities of boiling water. The residue was dehydrated with alcohol and dried *in vacuo*. The material contained 8·9 per cent. N and 22 per cent. agar, estimated according to the method of Sørensen and Haugaard (1933). A specimen of the agar purified by formamide treatment was used for colorimetric comparison. The protein preparation gave only a trace of colour with orcinol and sulphuric acid, under the conditions of estimation employed. If one may judge from the limited number of animals used, the removal of the excess agar enhanced the antigenicity of the agar protein complex. Three intravenous doses of 0·05 mg. now gave rise to powerful kanten precipitins in each of the 3 rabbits inoculated (Table I, animals 182, 183 and 184).

An antigenic complex was also prepared from agar and the conjugated protein obtained from the " O " antigen of *Bact. typhosum*. The same method for the formation of the complex was employed, and the resulting agar-protein complex was very similar in general appearance to that derived from agar and the " Shiga " protein component. The material again showed itself to be antigenic, and although three doses of 0·05 mg. proved insufficient to elicit

demonstrable kanten or agar precipitins, a further four similar doses gave rise to strongly precipitating sera (Table I, rabbits 185 and 186).

In view of the claim made by Zozaya (1932) that agar could be rendered antigenic by adsorption on collodion, the agar preparation employed in the experiments described above was treated with a preparation of collodion (Schering-Kahlbaum) particles prepared according to Zozaya's technique. The particles were readily agglutinated with anti-agar immune serum, thus indicating that an agar-collodion adsorption complex had been formed. The agar-coated collodion particles were given intravenously to two rabbits (Table I, animals 176 and 177). Four doses each of 0·2 mg. gave no demonstrable anti-agar immune-body. After a week's rest a further course of four doses of 0·5 mg. was given, but there was no evidence that even weak agar precipitins had been formed.

Preparation of kanten for use as test hapten.

The anti-agar sera from rabbits immunized with the agar-protein complex were found to give heavy precipitation with a preparation of partially degraded agar called kanten. The preparation and properties of the substance have been described in detail by Takahashi and Shirahama (1934) and by Pirie (1936). Kanten lends itself more readily to purification than agar since it is readily soluble in cold water or saline yielding clear mobile solutions, and for this reason it has been found very convenient to use kanten in place of agar as test hapten. A 1 per cent. solution of agar was heated at 100° for 2 hours, set aside to cool slowly and left overnight at 0°. The liquid which separated from the agar-gel was collected and after concentration was evaporated to dryness. The crude kanten obtained in this manner contained 1·7 per cent. N, showed a negative rotation, $[\alpha]_{5461}$ −47°, and dissolved readily in cold water to yield a clear solution. The material could be recovered from aqueous solution by precipitation with acetone, but precipitation was not complete even at 80 per cent. acetone level. A convenient method for the removal of nitrogenous impurities, however, was found and was based on the insolubility of kanten in 90 per cent. phenol solution. After three extractions with 90 per cent. phenol the nitrogen remaining in the kanten fell to 0·3–0·4 per cent. This material was used as test substance in place of agar in all experiments recorded in Table I.

The gum acacia antigen.

A method of purification of the commercial gum was chosen which was designed to avoid the use of strongly acid or alkaline reagents or heat treatment in order to reduce the risk of any degradation of the polysaccharide taking place. A sample of commercial gum B.P. (1 gm.) was dissolved in formamide (50 ml.), the small residue which remained undissolved was removed and the clear solution was treated with alcohol. The bulk of the material precipitated between the alcohol levels 40–50 per cent. Only a small quantity of substance was thrown out of solution above 50 per cent. alcohol and this was rejected. The main fraction was washed thoroughly with alcohol,

TABLE I.—*Immunization Experiments with Agar Antigen.*

Substance used for intravenous injections.	Animal number.	Dose i.v. First course.	Precipitation test. Dilution of kanten. 1:10^4.	1:10^5.	1:10^6.	Dose i.v. Second course.	Precipitation test. Dilution of kanten. 1:10^4.	1:10^5.	1:10^6.
Agar agar-"Shiga" conjugated protein complex (containing excess agar)	142	3 × 0·05 mg.	0	0	0	5 × 0·2 mg.	4	4	1
	143		0	0	0		4	4	1
Agar agar-"Shiga" conjugated protein . . .	182		1	2	0		2	2	0
	183	3 × 0·05 mg.	2	3	1	3 × 0·05 mg.	3	3	1
	184		3	4	0		4	4	1
Agar agar-"Typhosum" conjugated protein complex (containing excess agar)	185	3 × 0·05 mg.	0	0	0	4 × 0·05 mg.	3	3	1
	186		0	0	0		2	1	0
Agar-Agar formamide purified	140	3 × 0·05 mg.	0	0	0	5 × 0·2 mg.	0	0	0
	141		0	0	0		0	0	0
	170	4 × 0·2 mg.	0	0	0	6 × 0·2 mg.	0	0	0
	171		0	0	0		0	0	0
Agar agar (adsorbed on collodion particles) . .	176	4 × 0·2 gm.	0	0	0	4 × 0·5 gm.	0	0	0
	177		0	0	0		0	0	0

4, Very heavy precipitation. 3, Heavy precipitation. 2, Distinct precipitation. 1, Trace of precipitation. The numerals bear the same significance in this and subsequent tables.

dissolved in water and dialysed against repeated changes of distilled water. The acid polysaccharide was recovered by addition of an equal volume of ethyl alcohol after acidification with 1 ml. glacial acetic acid. The substance was redissolved in distilled water and again precipitated from solution by addition of an equal volume of alcohol. The dried preparation contained 0·23 per cent. N and 0·4 per cent. ash.

A group of 6 rabbits was inoculated with varying doses of the purified gum acacia as set out in Table II (animals 178, 179, 199, 200, 205 and 206), but no precipitins specific for gum acacia were detected in any of the sera examined. Uhlenhuth and Remy (1933, 1934) showed that certain commercial preparations of gum arabic are antigenic, but that after purification to remove a nitrogenous contaminant, the resulting gum no longer gave rise to immune-body. Similarly, Seideman (1940) showed that the serum of animals immunized with gum arabic preparations that gave positive protein reactions responded with the formation of immune-bodies reactive against a purified non-antigenic gum arabic.

The sample of " Shiga " conjugated protein used to prepare the gum acacia antigen was obtained from the bacterial antigen isolated by the phenol method (Morgan and Partridge, 1941*b*). The purification of the conjugated protein consisted of a single precipitation with acetic acid from solution in cold, dilute NaOH solution. The sample contained 11·2 per cent. N, 1·07 per cent. P, and showed $[\alpha]_{5461}$–48 $\pm$ 3°. The finely powdered protein (20 mg.) was mixed with the purified gum acacia (30 mg.) and formamide, m.p. + 2°, (1 ml.) was added. The mixture was thoroughly stirred and allowed to stand at 0–2° overnight. Solution was not quite complete ; the small residue was rejected, however, and the clear supernatant was transferred to another centrifuge tube and 4 ml. alcohol added. The precipitate of protein-gum acacia complex was washed three times with alcohol to effect the complete removal of formamide, and the substance was suspended in 10 ml. of 0·9 per cent. NaCl solution. An opalescent suspension of the complex was obtained which slowly settled on standing. The suspension was centrifuged and the clear supernatant discarded. The insoluble material contained 8·9 per cent. polysaccharide. It was again taken up in 10 ml. of 0·9 per cent. NaCl solution and finely dispersed by means of a glass tissue grinder. The protein content of the suspension was estimated and the volume adjusted so that the suspension contained 1 mg. of protein per ml. The dose given to the animals (Table II, animals 217, 221 and 227) was 0·5 ml.

A sample of the conjugated protein component of the " 0 " antigen of *Bact. typhosum* was also used in place of the " Shiga " conjugated protein as the second component in the gum acacia antigenic complex. The protein was prepared from a diethyleneglycol primary extraction product of a culture of the " 0 " strain " 901 " and contained 11·5 per cent. N. Details of its preparation and properties will be given in a later paper. The technique used to accomplish the combination was the same as that already described. The complex proved to be a powerful antigen, and three intravenous doses of 0·05 mg. (calculated as polysaccharide) only were sufficient to induce the formation of strong gum acacia precipitins in the two animals immunized (Table II, animals 197 and 198).

TABLE II.—*Immunization Experiments with Gum Acacia Antigen.*

Substance used for immunization.	Animal number.	Dose. First course.	Precipitation test. Dilution of gum acacia. 1:10⁴.	1:10⁵.	1:10⁶.	Dose. Second course.	Precipitation test. Dilution of gum acacia. 1:10⁴.	1:10⁵.	1:10⁶.
Gum acacia-"Typhosum" conjugated protein complex	197	3 × 0·05 mg.*	2	3	0	3 × 0·5 mg.*	3	4	1
	198		2	2	0		4	4	2
Gum acacia-"Shiga" conjugated protein complex	217		4	4	2		..	..	..
	221	3 × 0·05 mg.*	4	4	2	..	..	..	..
	227		4	4	2		..	..	..
Purified gum acacia	178		0	0	0		0	0	0
	179		0	0	0		0	0	0
	199		0	0	0		0	0	0
	200	3 × 0·05 mg.	0	0	0	3 × 0·5 mg.	0	0	0
	205		0	0	0		0	0	0
	206		0	0	0		0	0	0

* Dose calculated as pure gum acacia injected.

TABLE III.—*Immunization Experiments with Cherry Gum Antigen.*

Substance used for immunization.	Animal number.	Dose. First course.	Precipitation test. Dilution of cherry gum. 1:10⁴.	1:10⁵.	1:10⁶.	Dose. Second course.	Precipitation test. Dilution of cherry gum. 1:10⁴.	1:10⁵.	1:10⁶.
Cherry gum-"Shiga" conjugated protein complex	232	3 × 0·05 mg.*	3	2	1	3 × 0·05 mg.*	3	4	2
	262		4	2	1		4	4	2
	263		4	2	1		3	4	2
Purified cherry gum	235		0	0	0		0	0	0
	243	3 × 0·05 gm.	0	0	0	3 × 0·5 mg.	0	0	0
	257		0	0	0		0	0	0
	272		0	0	0		0	0	0

* Dose calculated as pure cherry-gum.

Cherry-gum antigen.

A specimen of cherry-gum (0·75 mg.) kindly supplied by Prof. J. R. Marrack was dissolved in water (25 ml.). A small insoluble residue was removed, and the gum was precipitated from solution by the addition of 50 ml. alcohol containing a few drops of glacial acetic acid. The material was thoroughly washed with absolute alcohol and dried *in vacuo*. The gum contained 0·52 per cent. N and was further purified by extraction with 90 per cent. phenol solution. The powder swelled to a clear gelatinous mass but did not dissolve. After three extractions the phenol was removed from the gum by trituration with alcohol. The material after drying *in vacuo* weighed 0·46 g. and was free from N. The antigenic complex was prepared exactly as described above for the gum acacia antigen. The complex after precipitation from formamide with alcohol was taken up in saline, vigorously shaken and centrifuged. The deposit was again shaken with saline and finely dispersed with the aid of a glass tissue grinder. The resulting suspension was not stable, but slowly settled out on standing overnight in the cold room. Four rabbits were given six intravenous doses of the purified cherry-gum (Table III, animals 235, 243, 257 and 272), and three animals (Nos. 232, 262 and 263) were given the equivalent amount of gum in the form of cherry-gum protein complex. It will be seen from the results given in Table III that the hapten alone failed to induce the formation of specific cherry-gum precipitins, whereas the complex caused the production of specific immune-body after three doses only.

Cross Precipitation Tests.

In view of the overlapping specificity shown by the partial hydrolysis products of certain vegetable gums with anti-pneumococcal serum (Type II) (Marrack and Carpenter, 1938), it was considered of interest to investigate the reactions of the anti-sera described above with the pneumococcal specific polysaccharides (Types I and II) and with the heterologous gums. The undiluted immune serum was mixed with an equal quantity of a dilution of the gum or polysaccharide preparation, incubated for 2 hours at 37° and examined after standing at 0° for 18 hours. The results of these tests are given in Table IV. Gum acacia failed to give any precipitate when mixed

TABLE IV.—*Showing the Specificity of the Anti-polysaccharide Immune Sera.*

Test hapten.	Precipitation test with—								
	Anti-agar serum. Dilution of test hapten.			Anti-gum acacia serum. Dilution of test hapten.			Anti-cherry gum serum. Dilution of test hapten.		
	1 : 10^4.	1 : 10^5.	1 : 10^6.	1 : 10^4.	1 : 10^5.	1 : 10^6.	1 : 10^4.	1 : 10^5.	1 : 10^6.
Kanten	4	4	1	1	0	0	0	0	0
Gum acacia	0	0	0	4	4	2	0	0	0
Cherry gum	0	0	0	1	2	1	3	4	2
Gum tragacanth	0	0	0	0	0	0	0	0	0
Hyaluronic acid	0	0	0	0	0	0	0	0	0
Pneumococcus specific polysaccharide Type I	1	1	0	0	0	0	0	0	0
Pneumococcus specific polysaccharide Type II	0	0	0	0	0	0	0	0	0
Gastric mucin "A" substance	0	0	0	0	0	0	0	0	0

with an anti-cherry-gum serum, whereas cherry-gum gave distinct, although weak, precipitation with anti-gum acacia serum. Preparations of kanten, gum tragacanth, hyaluronic acid, the specific polysaccharides of pneumococcus (Types I and II) and the blood group "A" polysaccharide isolated from pig gastric mucin failed to give any precipitation. The slight precipitation given by the Type I pneumococcus hapten with the anti-agar serum was probably due to agar substance in the preparation of the specific substance.

Complement-Fixation Tests.

Complement-fixation tests were carried out with anti-sera prepared against agar, gum acacia and cherry gum and the corresponding polysaccharide haptens. The soluble degradation product of agar, kanten, was employed as hapten in the tests with anti-agar serum, but owing to its strong anti-complementary action up to a dilution of 1 : 100,000 the tests have been considered untrustworthy. Similarly, with the cherry-gum-anti-cherry gum system the tests were rendered unsatisfactory owing to the anticomplementary action, up to a dilution of 1 : 200, shown by anti-cherry gum serum. On the other hand neither the gum acacia at 1 : 1000 dilution nor its homologous serum at 1 : 20 dilution showed any anticomplementary action, and the complement-fixation tests showed that 1 M.H.D. of complement (0·2 ml.) was fixed by as little as 1 part of gum in 1 : 10,000,000 parts of saline (0·2 ml.) in the presence of a 1 : 20 dilution of the immune-body (0·2 ml.). Anti-sheep cell serum, 3–5 M.H.D., was used as amboceptor throughout the tests. Owing to the unsatisfactory nature of the agar and cherry gum complement-fixation tests no cross-reactions within the three systems were investigated.

Anaphylaxis Experiments.

Groups of guinea-pigs (300–450 g.) were passively immunized with anti-agar, anti-gum acacia and anti-cherry gum rabbit serum by receiving an intraperitoneal injection of 2–3 ml. of the immune serum. After 24–36 hours each animal was given an intravenous dose of the homologous hapten and observed for symptoms of anaphylaxis. Three animals immunized with anti-agar serum showed severe anaphylaxis after a dose of 1 mg. kanten; one animal died after 2 minutes. Two other animals which received 0·1 mg. of kanten showed definite symptoms of anaphylaxis, but subsequently recovered. Five guinea-pigs passively sensitized with 2 ml. of anti-gum acacia serum and shocked with 1 mg. of the homologous gum showed severe anaphylaxis. Three of the animals died in 2–3 minutes after receiving the injection; the remaining two animals slowly recovered from shock during the following 2–3 hours. Similarly, a group of guinea-pigs, each of which had received an intraperitoneal injection of 3 ml. of anti-cherry gum rabbit serum likewise showed all the usual symptoms of shock when given an intravenous dose of the homologous gum 24 hours after the serum. Owing to the limited amounts of immune sera that were available no anaphylaxis tests that involved the use of heterologous haptens to induce shock were made.

DISCUSSION.

The method of combining bacterial polysaccharide and protein components in formamide solution to form antigenic complexes has now been extended to include the formation of antigens in which polysaccharides of vegetable origin function as haptens. In this manner the polysaccharides agar-agar, gum acacia and cherry gum have been combined with the conjugated protein components of the " 0 " somatic antigens of *Bact. shigae* and *Bact. typhosum* and powerful antigenic complexes have been formed.

Agar, gum acacia and cherry gum do not possess the property of forming soluble complexes with the conjugated protein in aqueous solution in the same way as certain other bacterial and tissue haptens (Morgan and Partridge, 1941*b*; Morgan, 1941). It is now shown that when these gums are mixed in formamide solution with a sample of the bacterial protein, the protein firmly retains part of the polysaccharide, yielding a complex that cannot be altered greatly in composition by repeated washing with water or dilute salt solution. Although the polysaccharide content of the association complex is in each instance low compared with that of the natural bacterial antigens, the complexes are nevertheless found to induce the formation of powerful anti-gum immune sera on injection into rabbits. It is now well known that proteins when combined with mono- and di-saccharides, polysaccharides and many simple organic compounds (see Landsteiner, 1936; Marrack, 1938) acquire a new immunological specificity, and when the complexes are subsequently injected into animals they induce the formation of immune body that is largely specific for the attached group. It is, however, frequently difficult to secure a chemical linking of protein with the more complex and labile polysaccharides such as the specific acetyl polysaccharide of the pneumococcus (Type I) or the " A " specific blood group polysaccharide of gastric mucin or saliva, without damaging the polysaccharide molecule and thus modifying its immunological specificity. For this reason a method of forming an antigenic polysaccharide-protein complex that involves a procedure no more violent than solution of the components in anhydrous formamide at room temperature is likely to prove of considerable value in future investigations.

Some years ago Sordelli and Mayer (1931) found that the immune-sera of horses that had been immunized with bacteria grown on agar medium developed agar precipitins. Utilizing this observation, Morgan (1936) showed that it was possible to detect, by means of anti-agar horse serum, agar and agar breakdown products in a number of specific polysaccharide preparations. The potency of these anti-agar horse sera, however, is not high even after the horses have received a prolonged course of immunization lasting over a period of several months. Furthermore, such sera arise only occasionally, and must be considered as accidental by-products in the manufacture of therapeutic sera for clinical use. The potent anti-agar rabbit serum, on the other hand, produced by means of the artificial agar-protein complex has proved of considerable value in following the removal of agar and kanten from antigenic material isolated from agar grown bacteria. Indeed, the precipitation reaction

of agar or kanten with its homologous immune-body is so sensitive that material which fails to yield any of the colour reactions in the tests elaborated by Pirie (1936) for the detection of agar will nevertheless give definite precipitation when tested with anti-agar serum. The strong anti-complementary properties of kanten suggest that the anti-complementary nature of certain bacteria grown on an agar medium may, in part, be due to a surface coating of kanten. Since the original observation of Sordelli and Mayer no explanation for the conversion of agar into an antigenic complex has been forthcoming, but in view of our observations it seems likely that agar or its breakdown products combines with certain protein components of the bacterial cell and in this manner is transformed into a full antigen.

The anti-gum acacia and anti-cherry gum immune sera were considered suitable for studying the serological relationships that might exist between these sera and other heterologous haptens, such as the specific polysaccharides of pneumococcus (Types II and III), thus contributing to the valuable observations of Marrack (1937) and of Marrack and Carpenter (1938) on the cross reactions shown by the acid hydrolysis products of vegetable gums and pneumococcus (Types II and III) immune-body (see also Morgan, 1937). Heidelberger, Avery and Goebel (1929) showed that the native gum acacia reacted only occasionally, and then very weakly, with Type II and III anti-pneumococcus serum, and that it was necessary to partially hydrolyse the gum before it showed any pronounced cross-reactions with the pneumococcus Types II and III immune-body. These workers suggested that even the slight activity of the original gum might be satisfactorily accounted for on the assumption that traces of the specifically reacting material were formed during a period of exposure in the process of refining the commercial product or by enzyme action. Indeed, it is clear from our experiments that there is no cross precipitation reaction between the specific polysaccharide hapten of the pneumococcus (Type II) and gum acacia and gum cherry immune-body. It seems improbable therefore that the aldobionic acid structure, which plays such an important part in the cross reactions shown by the partially hydrolysed gums and the pneumococcus (Type II) immune-body, exists as a serologically important configuration in the native gum-acacia and cherry-gum. This conclusion is supported by the observation that glucuronic acid fails to inhibit either of the homologous gum-anti-gum precipitation reactions. Galacturonic acid is also inactive in similar inhibition tests. Furthermore, virulent cultures of pneumococcus Types II and III are not agglutinated by the gum acacia and cherry gum immune-body.

Cretcher and Butler (1928) and Butler and Cretcher (1929) showed that by mild acid hydrolysis gum acacia loses its arabinose and rhamnose components very readily. Under these conditions of hydrolysis it is known from the work of Heidelberger, Avery and Goebel (1929) that a new serological specificity develops and that the resulting partially degraded gum now reacts with pneumococcus (Types II and III) antibody. These observations suggest that the serological specificity of the native gum acacia might be due in part to the specific configuration of the arabinose and rhamnose molecules, especially if the ring structure of the complex acidic nucleus brought forward by Norman (1937) is accepted. In such a structure for the native gum the

arabinose and rhamnose units are joined to the nucleus by glucosidic linkage, and thus, by masking the reactive surface of the aldobionic acid molecule they might be expected to play a dominant role in determining the serological specificity of the native gum. It has been found, however, that *d*-arabinose, *l*-rhamnose and *d*-fucose fail to inhibit the acacia-anti-acacia or the cherry-gum-anti-cherry gum precipitation reactions. There is, therefore, no evidence based on specific inhibition that the pentose and methyl pentose components of the natural gums play any dominant part in determining their immunological specificity. We have had no opportunity of investigating further many points of considerable interest arising out of this work.

SUMMARY.

1. The formation of antigenic complexes from the non-antigenic polysaccharides, agar, gum acacia and cherry gum and the conjugated protein component of the specific somatic antigens of *Bact. shigae* and *Bact. typhosum* are described.

2. The artificial antigens induce the formation of immune-body specific for the polysaccharide components of the artificial antigenic complexes.

3. The homologous gum-anti-gum precipitation reactions are not inhibited by glucuronic or galacturonic acid and the anti-gum sera fail to agglutinate virulent pneumococci (Types II or III).

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BACTERIAL INHIBITION BY METABOLITE ANALOGUES. 3. PANTOYLTAURINE. THE ANTIBACTERIAL INDEX OF INHIBITORS.

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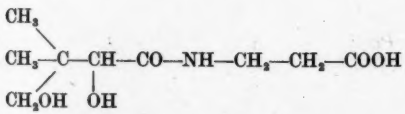
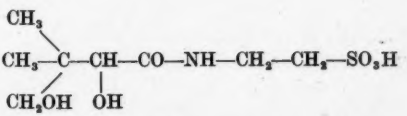
SEVERAL inhibitors of bacterial growth have already been designed by altering in a defined manner the structures of substances which are necessary for growth. Inhibitory analogues of nicotinic acid (McIlwain, 1940) and of some aliphatic amino acids (McIlwain, 1941) have been prepared in this way. The alteration has consisted of the substitution of $-SO_3H$ or $-SO_2NH_2$ for a carboxylic grouping in the essential substance, and was first based on Woods' (1940) finding of the functional relation between sulphanilamide ($R.SO_2NH_2$) and *p*-aminobenzoic acid (R.COOH). These investigations have now been extended to pantothenic acid, which is essential to many pathogens whose growth it is frequently desirable to limit. The analogue pantoyltaurine has



Pantothenic acid (=pantoyl-β-alanine).



Pantoyltaurine.

been found extremely inhibitory to three such organisms by virtue of its relation to pantothenate. Optimal conditions of *in vitro* growth have been used in studying the susceptible organisms. The inhibitory power has been expressed quantitatively as an antibacterial index which enables antibacterial action *in vivo* to be anticipated.

EXPERIMENTAL.

Technique.

In testing, bacteria were grown from inocula of about 1000 organisms added to media containing the substances investigated and made to a final

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volume of 10 ml. Cultures were incubated at 37° in 5 per cent. CO_2-air, and the extent of growth was judged visually, the + signs of the tables being roughly proportional to its mass. Sodium (+)pantothenate was autoclaved as a $M/100$ solution made from the calcium salt given by Messrs. Merck & Co., Inc. Sodium-*dl*-pantoyltaurine was prepared by J. W. Barnett and F. A. Robinson, of Glaxo Laboratories, Ltd.; it has been prepared independently by Snell (1941). A $M/100$ solution was filtered to sterilize; it has since been found stable to autoclaving at neutral pH, but not at pH 2–3. The products of its breakdown were not inhibitory.

Inhibition of Streptococcus haemolyticus.

Five strains of groups A and C streptococci were grown in a hydrolysed casein medium with the addition of glucose and other nutrients. This medium can be made free from pantothenate, and had previously been used to demonstrate the need of the organisms for that compound (McIlwain, 1939). The present strains (which included the N.C.T.C. organisms 5493 and 5631) also required pantothenate, growth with $10^{-7}M$ and more being optimal. Maximum growth was uniformly reached in 18 hours from the small inoculum which was taken from a 16–24-hour serum-agar slope; with less pantothenate growth fell as indicated in Table I, A, and was not detectable with $10^{-9}M$ of the acid.

TABLE I.—*Growth and Inhibition of* Streptococcus haemolyticus.

	Addenda.		Growth after (hours and days).				
	Pantothenate, $M \times 10^7$.	Pantoyltaurine, $M \times 10^5$.	18–20.	26–28.	2.	4.	7.
A.	0	0	0	0	0	0	0
	1 to 1000	0	++++				
	0·2	0	+++	++++			
	0·04	0	++	++	++	++	++
	0·008	0	0	0	0	0	0
B.	1	2 to 100	0	0	0	0	0
	1	1	0	0	+	++++	
	1	0·4	+	+++	++++		
	1	0·2	++	+++	++++		
	1	0·08	++++				
C.	250	50	++++				
	50	50	+++	++++			
	10, 2	50	0	0	0	0	0
	50	10	++++				
	10	10	+++	++++			
	2	10	0	0	0	0	0
	10	2	++++				
	2	2	+++	++++			
	0·4	2	0	0	0	0	0
	2	0·4	++++				
	0·4	0·4	++	+++	++++		
	0·08	0·4	0	0	0	0	0
	0·4	0·08	+++	++++			
	0·08	0·08	+	+	++	++	
	0·016	0·08	0	0	0	0	0

Pantoyltaurine prevented or limited the growth of all strains examined. Table I, B, shows that in the presence of optimal amounts of pantothenate $2 \times 10^{-6}M$ and more pantoyltaurine restrict growth, while $2 \times 10^{-5}M$ and more completely prevent it. The same concentrations were effective anaërobically against the strain so investigated.

Relation of inhibition to pantothenate concentration.

Pantoyltaurine was designed to inhibit organisms by limiting their use of pantothenate. That such is its action was shown by increasing the concentration of pantothenate in the basal medium to a level higher than that normally required for optimal growth. Such increase permitted growth in the presence of the previously inhibitory $2 \times 10^{-5}M$ pantoyltaurine, but growth could again be prevented by more pantoyltaurine. Several such experiments are recorded in Table I, C. From these results, the action of a given quantity of pantothenate can be described approximately as the reduction of the effective concentration of pantothenate to a fraction of that actually present. With $10^{-4}M$ pantoyltaurine this fraction is about 1/100th. Considering the whole of Table I, C, it will be seen that a given mass of growth is produced, not by a given quantity of pantothenate, but by a given ratio of concentrations of pantoyltaurine and pantothenate. When this ratio is 500 : 1 no growth occurs, when it is 100 : 1 growth is suboptimal, and when it is 20 : 1 growth is usually normal.

The interrelations observed between growth-promoting and inhibiting agents have so far fallen into the types described previously (McIlwain, 1940), and the characteristics described above are those of the Type I, previously exhibited by, e.g., pyridinesulphonamide-inhibition of nicotinamide-promoted staphylococcus growth. The delayed growth of streptococci in the presence of limiting concentrations of pantoyltaurine (e.g. $10^{-5}M$ of Table I, B) suggested that the compound was bacteriostatic rather than bactericidal. After 24 hours' incubation in media containing totally inhibitory concentrations of pantoyltaurine, the addition of excess pantothenate caused prompt growth : the effect of the inhibitor during this period is therefore mainly bacteriostatic, and reversible.

Specificity of relation to pantothenate.

Pantothenate clearly reduces the inhibitory effects of a given quantity of pantoyltaurine, and from the point of view of the possible therapeutic use of pantoyltaurine, it is important to find whether other materials have such action. The culture medium used in testing already contains most of the substances known to be important in bacterial growth. The addition of higher concentrations of 14 amino acids, of biotin, choline, and *p*-aminobenzoic acid did not alter the inhibitory power of pantoyltaurine. The components of pantothenic acid, β-alanine and pantoic acid (α-γ-dihydroxy-β.β-dimethylbutyric acid) were without effect on the inhibition in concentrations up to $10^{-3}M$.

The antagonists of greatest practical importance are those of animal

tissues or fluids. These have been found to have limited powers of antagonizing pantoyltaurine. In Table II their ability to reverse such inhibition is compared with the amount of pantothenate which they contain, as determined by straightforward bacterial growth tests. The final column of the table gives the ability of the materials to antagonize pantoyltaurine, in terms of their pantothenate content. The constancy, within the limits of accuracy

TABLE II.—*Pantothenate as the Main Constituent of Normal Tissues which Antagonizes Pantoyltaurine.*

Material.	(*a*) Pantothenate content (*M*).*	(*b*) Concentration (% in medium) just reversing the inhibition of $2\times10^{-4}M$ pantoyltaurine.*	(*a*) × (*b*), *i.e.*, the pantothenate content (*M*) of a solution just reversing $2\times10^{-4}M$ pantoyltaurine.
(+)Pantothenate (control)	10^{-5}	2	2×10^{-7}
Hot extract of ox-heart (1g./ml.)	5×10^{-5}	1	5×10^{-7}
Na_2SO_4 extract† of ox-heart (2·5 g./ml.)	5×10^{-5}	1	5×10^{-7}
Serum (horse)	4×10^{-6}	5	2×10^{-7}
Plasma (human)	2×10^{-6}	>5; <25	10^{-7}–5×10^{-7}
Urine (human)	10^{-5}	2	2×10^{-7}

* Test organism: *streptococcus*, grown as described in the text.
† Prepared according to Deutsch *et al.* (1938).

of the experiments, of these values shows that pantothenate is the main agent present in normal tissues which antagonizes pantoyltaurine. The presence of other antagonists would have been shown by a value of $<2\times10^{-7}$. This result is a further indication of the specificity of pantoyltaurine action, as such extracts contain many identified and unidentified growth-promoting agents. But unless such substances possess the specific growth-promoting powers of pantothenate, they do not contribute to pantoyltaurine reversal.

Diplococcus pneumoniae.

Conditions of growth.

Seven strains representative of Types I, II and III (including N.C.T.C. 2426, 2427, 5485 and 5486) did not grow from the standard inoculum in media prepared as closely as possible to that of Rane and Subbarow (1940), nor after the addition of most other compounds known to improve bacterial growth. Less defined media were therefore used. Poor growth was obtainable under the conditions used for streptococcus, and this could be made optimal by the addition of a little filtered plasma or yeast extract. The former was chosen as the quantity necessary for optimal growth added less pantothenate to the medium. In the presence of the minimal optimal quantity of plasma (2–5 per cent.) pantothenate was necessary for growth and $10^{-7}M$ was optimal. β-Alanine and pantoic acid did not replace pantothenate. As suggested by Dr. D. D. Woods, inocula from 6-hour serum-broth cultures of pneumococci gave more dependent results than older, possibly lysed, cultures. Under testing conditions, growth in uninhibited controls was much heavier than in serum-broth and was of typical diplococci without chain forms. These, and rapid lysis, appeared to be associated with suboptimal nutrition.

Inhibition.

All strains were regularly susceptible to practically the same degree, $10^{-4}M$ pantoyltaurine preventing growth in the presence of $10^{-7}M$ pantothenate. Two strains were examined in detail and found to exhibit quantitative interrelationships between the concentrations of the two substances similar to those recorded in Table I, C, for streptococcus. The lower concentrations of the table were not investigated as the concentration of pantothenate contributed by the plasma of the medium was of this order. Pantoyltaurine in 1000 times the quantity of the pantothenate of the medium inhibited, but in 200 times the quantity did not. β-alanine and pantoic acid were ineffective in reversal, and the antagonizing action of plasma was wholly explicable in terms of its pantothenate content.

Corynebacterium diptheriae.

Conditions of growth.

The medium used was similar to that of Mueller (1939), and was prepared from the hydrolysed casein basal medium (McIlwain, 1939) with the following additions per 10 ml.: glucose 25 mg., cystine and methionine 0·25 mg., tryptophane 0·1 mg., pimelic acid 50μg., nicotinamide 12·5μg., aneurin 0·3 μg., $MgSO_4$ 0·4 mg., and $Fe(NH_4)_2(SO_4)_2.6\ H_2O$, 0·15 mg. Of the strains investigated, two grew maximally on addition of β-alanine to this medium; the growth of two others was further improved by the addition of pantothenate also, while for one organism pantothenate was essential. The degree of pantothenate need was not correlated with the type of organism (*cf.* Evans *et al.*, 1939).

TABLE III.—*Growth and Inhibition of* C. diphtheriae *Strains.*

Strain.	Addenda. Pantothenate, $M \times 10^7$.	Addenda. Pantoyltaurine, $M \times 10^6$.	Growth after (days). 1.	2.	5.
G1, *mitis*	0; or β-alanine, 10	0	0	0	0
	1 to 100	0	+++	++++	
	0·2	0	++	+++	+++
	0·04	0	+	++	+++
	50	50	++	++++	
	10, 2	50	0	0	0
	10	10	0	+++	++++
	2	10	0	0	0
	2	2	0	+	+++
	0·4	2	0	0	0
GW1, *gravis*	0	0	0	0	0
	0 + β-alanine, 100	0	+	+++	++++
	0·2	0	++	++++	
	0·2	50, 10	0	0	0
	0·2	1	+	++++	
R7 (*mitis*) and	0	0	0	0	0
R20 (*gravis*)	0 + β-alanine, 100	0	+	++++	
	0·2	0	+	++++	
	0·2	50, 5	+	++++	

G1156, *mitis*, grew slowly with β-alanine in place of pantothenate, and was inhibited to the same degree as GW1.

Inhibition.

Table III shows an immediate correlation between the need of a strain for pantothenate and its inhibition by pantoyltaurine. One *mitis* strain, which would not grow without added pantothenate, was inhibited to approximately the same extent as were the streptococci. Its responses to mixtures of pantothenate and pantoyltaurine were also similar. One *mitis* and one *gravis* strain whose growth were improved by, but did not demand, pantothenate were inhibited to a lesser degree, while one *gravis* and one *mitis* strain which did not need pantothenate were not inhibited.

Inhibition of all susceptible strains, including the exacting *mitis* strain in certain cases, could be reversed by β-alanine as well as by pantothenate. Non-exacting strains have been shown by Evans *et al.* (1939) to synthesize substances of pantothenate growth-promoting activity from β-alanine. Pantoic acid was ineffective in reversal.

Organisms Not Inhibited.

Bact. coli, in a simple ammonium lactate medium, and *Proteus vulgaris*, in that medium with the addition of nicotinamide (Fildes, 1938), were not inhibited by up to $2\times10^{-3}M$ pantoyltaurine. Growth of neither was affected by pantothenate. *Proteus morgani* in an amino acid medium similar to that of Pelczar and Porter (1941) required added pantothenate but was not inhibited; that this was not due to any peculiarity of the conditions of growth was shown by growing it in the casein medium used for streptococci, when again it was not inhibited. Growth of *Staphylococcus aureus* in an amino acid medium (Gladstone, 1937) was improved by added pantothenate but the organism was not inhibited by pantoyltaurine. Pantoyltaurine did not replace pantothenate in growth of *P. morgani* or of staphylococcus.

DISCUSSION.

Mode of action of pantoyltaurine.

Pantothenate is a growth factor for all organisms found susceptible to pantoyltaurine inhibition (see above, and also Snell, 1941), and as such is considered to take part in processes essential to their growth. Such processes are probably enzymic, and pantoyltaurine appears to have about 1/100th–1/500th of the affinity of pantothenate for the enzymes concerned. In concentrations greater than 100–500 times that of the pantothenate present, pantoyltaurine is pictured as occupying the majority of the active centres of the enzymes, having displaced the pantothenate normally reacting there. As it cannot functionally replace pantothenate, the essential process ceases and the organism cannot grow further. The quantitative interrelations of Table I, C, are typical of such a competitive enzyme inhibition. Organisms needing pantothenate are not equally susceptible to pantoyltaurine; this may be explained in terms of their different needs for the products of the hypothetical inhibited enzymes, or different susceptibilities of the enzymes themselves (*cf.* Rona and Petow, 1924). Such difference in susceptibility affords an important basis for the differential action of inhibitors which is necessary in chemotherapeutic agents.

The antibacterial index.

The present method of investigation of inhibitory agents yields in cases such as pantoyltaurine a quantitative measure of their inhibitory power which is important in relation to their possible therapeutic use. It is proposed to term the ratio C_I/C_M, where C_I is the concentration of an inhibitor just bacteriostatic in the presence of a concentration C_M of the corresponding metabolite, the *antibacterial index* of the inhibitor with respect to the organism tested. Such ratios have been shown to be constant within the limits of experimental accuracy over a wide range of concentrations in the case of pantoyltaurine; similar observations were made with pyridine-3-sulphonamide (McIlwain, 1940), and have now been frequently recorded with sulphanilamide (Woods, 1940; Rubbo and Gillespie, 1942). The values for these ratios (the corresponding metabolite being given in parentheses) are: The antistaphylococcal index of pyridine-3-sulphonamide (nicotinamide), 250,000; antistreptococcal index of sulphanilamide (*p*-aminobenzoic acid), 25,000; antipneumococcal index of pantoyltaurine (pantothenate) 1000, and its antistreptococcal index, 500. These figures show pantoyltaurine to be a very effective inhibitor. Allowing for the probable inactivity of the (–)isomeride present in the *dl*-material used (Snell, 1941), the antistreptococcal index of (+)pantoyltaurine becomes 250. By using data such as that of Table II for the concentration of pantothenate in animal tissues, the concentration of pantoyltaurine necessary to inhibit streptococcus *in vivo* can be calculated by means of the antibacterial index. This index forms part of the chemotherapeutic index of Ehrlich: if a similar ratio C_H/C_M (C_H = concentration of drug just not damaging the most susceptible vital tissue of the host) is called the *host index* of the same drug, then assuming a common environment to the two types of cell, the chemotherapeutic index is given by *host index/antibacterial index*. It has been demonstrated (McIlwain, unpublished) that the antistreptococcal action of sulphanilamide *in vivo* is consistent with its antistreptococcal index which was determined *in vitro*. To antagonize streptococci in a tissue containing $2\times10^{-6}M$ pantothenate, $10^{-3}M$ pantoyltaurine is necessary; 1000 ml. of $10^{-3}M$ pantoyltaurine contain 0·25 g. As at least 2·5 g./kg. (private communication from F. A. Robinson) can be fed to mice without ill effects, it may be anticipated that an antistreptococcal level can be maintained in their blood-stream. The compound is extremely soluble in water.

SUMMARY.

As pantothenate is necessary for the growth of many pathogens, their growth might be prevented by limiting their use of pantothenate. This has been achieved *in vitro*, without reducing the pantothenate to below the level found in normal tissues, by addition of pantoyltaurine. It was anticipated that pantoyltaurine would be inhibitory, because it is structurally related to pantothenate in the same way that other inhibitors are to other growth essentials. Pantoyltaurine apparently acts by virtue of this structural relation, becoming attached to the organisms at enzymes where pantothenate must react for normal activity and growth. It has, however, less affinity for the

enzymes than has pantothenate, and a large excess of pantoyltaurine is necessary to block them. This is expressed in its antistreptococcal index of 500 (500 times the pantothenate concentration being necessary to stop growth of streptococci) and antipneumococcal index of 1000. These ratios, however, compare favourably with those of, e.g., sulphanilamide, and the normal concentration of pantothenate is sufficiently small for antistreptococcal activity to be anticipated *in vivo*.

I am greatly indebted to Mr. D. E. Hughes for assistance throughout this work, and to Miss M. Pownall for re-typing the strains of *C. diphtheriae* used, which were from a collection made by Dr. G. P. Gladstone.

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